UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE VETERINARIA

Departamento de Bioquímica y Biología Molecular IV

TESIS DOCTORAL

Identificación y caracterización inmunológica preclínica de antígenos con potencial vacunal frente a la malaria en un modelo de malaria murina.

Identification and preclinical immunological characterization of potential malaria vaccine antigens in a murine model of malaria.

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

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IDENTIFICATION AND PRECLINICAL IMMUNOLOGICAL CHARACTERIZATION OF POTENTIAL MALARIA VACCINE ANTIGENS IN A MURINE MODEL OF MALARIA

ALI NAGHI KAMALI

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UNIVERSIDAD COMPLUTENSE
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CERTIFICAN:

Que la memoria adjunta, titulada "……" ha sido realizada por el licenciado en Veterinaria y Master en Investigacion en Ciencias Veterinarias **Ali Naghi Kamali** bajo la dirección conjunta de los que suscriben, y cumple las condiciones exigidas para optar al título de Doctor por la Universidad Complutense de madrid.

Dr. Jose Manuel Bautista Santa Cruz **Dr. Antonio Puyet Catalina**

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To my parents

ABBREVIATION

Ab	Antibody
Ag	Antigen
AMA	Apical merozoite surface antigen
ATP	Adenosine 5′-triphosphate
BiP	Binding immunoglobulin protein
CFA	Complete Freund´s adjuvant
CHAPS	3-[(3-cholamidopropyl)dimethylamonio]-1-propanesulfonate
CSP	Circumsporozoite protein
DC	Dendritic cells
DNPH	2,4 dinitrophenylhydrazine
1D	One-dimensional
2D	Two-dimensional
EBA	Erythrocyte binding antigen
ECM	Experimental Cerebral Malaria
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
ES	Electrospray
Fab	Fragment antigen-binding
Fc	Fragment crystallizable
GRP	Glucose-regulated protein
4-HNE	4-hydroxy-2-nonenal
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
HSP	Heat shock protein
IFA	Incomplete Freund´s adjuvant
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IP	Immunoprecipitation

IRBC	Infected red blood cells
Kb	Kilobase
KDa	Kilodalton (molecular mass)
MAb	Monoclonal antibody
MALDI-MS	Matrix-assisted laser desorption/ionization–mass spectrometry
MALDI-TOF/TOF	Matrix-assisted laser desorption/ionization time-of-flight/time-
MS	of-flight mass spectrometry
MEGA10	N-Decanoyl-N-methylglucamine
MHC	Major histocompatibility complex
MS	Mass spectrometry
MS/MS	Mandem mass spectrometry
MSP	Merozoite surface protein
NCBI	National Center for Biotechnology Information
mM	Milimolar
MW	Molecular weight
NK	Natural killer cells
PABA	*P*-Aminobenzoic Acid
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PDI	Protein disulfide isomerase
Pi	Post infection
PMF	Peptide mass fingerprinting
PVDF	Poliviniliden-difluoruro
p.yy	*Plasmodium yoelii yoelii*
RAP	Rhoptry-associated protein
RBC	Red blood cell
RESA	Ring-infected erythrocyte surface antigen
ROS	Reactive oxygen species
rpm	Revolutions per minute
SDS	Sodium dodecyl sulfate
TCR	T cell receptor

TEMED	N,N,N',N'-tetramethylethylenediamine
TLRs	Toll-like receptors
Th	T helper cells
TNF	Tumor-necrosis factor
Tris	Tris(hydroxymethyl) aminomethane
WHO	World Health Organization

RESMEN

A pesar de los esfuerzos realizados durante más de un siglo en la investigación para suprimir la malaria, esta enfermedad sigue siendo una amenaza importante y creciente para la salud pública y el desarrollo económico de países en las regiones tropicales y subtropicales del mundo. La malaria humana está causada por la infección de parásitos intracelulares del género *Plasmodium* que se transmiten por mosquitos *Anopheles*. De las cinco especies de *Plasmodium* que infectan a seres humanos, las infecciones causadas por *Plasmodium falciparum (P. falciparum)* son las que muestran mayor tasa de mortalidad. Debido al incremento en la resistencia de *P. falciparum* a fármacos antimaláricos, estudios recientes sugieren que el número de los casos de la malaria puede doblarse en 20 años (WHO, 2007). El descubrimiento de una vacuna contra malaria representa por lo tanto una necesidad médica urgente para la extensa población que vive en áreas donde la malaria es endémica. La vacuna ideal contra esta enfermedad debería tener un coste de producción bajo, ser extremadamente segura y capaz de inducir inmunidad a largo plazo. Igualmente sería deseable que la vacuna fuera activa contra todas las cepas del parásito, dando lugar a la interrupción casi completa del ciclo vital de la malaria por la respuesta inmune inducida por la vacuna. Aunque se han obtenido resultados prometedores, en particular con vacunas de subunidades, el progreso de hacer una vacuna de la malaria se ha visto obstaculizado debido en parte a la extensa diversidad genética de los antígenos candidatos para la vacuna. En la vacunación por subunidades, los antígenos, parciales o completos, se identifican a partir del complemento proteómico con el objetivo de inducir inmunidad protectora frente al patógeno completo tras la vacunación. Durante el ciclo vital del *P. falciparum*, varios antígenos polimórficos del *parásito* se exponen al sistema inmune humano. Entre éstos, el antígeno-1 de la membrana apical (AMA1), la proteína de superficie del merozoito (MSP-1) y la proteína del circumesporozoito (CSP) son los más estudiados para el desarrollo de una posible vacuna. Los datos epidemiológicos sugieren que los anticuerpos adquiridos naturalmente contra las proteínas del merozoito (AMA1 y MSP1) contribuyen a la adquisición de la inmunidad protectora en áreas endémicas de malaria. Sin embargo, la alta variedad de polimorfismos antigenicos en estas proteínas continúan frustrando su uso en vacunas.

La inmunidad naturalmente adquirida, tanto en humanos como en modelos animales puede ser una herramienta valiosa para el desarrollo de una vacuna capaz de prevenir totalmente la infección, o bien la enfermedad severa y muerte. Sin embargo, los antígenos y los epítopos específicos que participan a la protección son en gran parte desconocidos, y su correlación con la protección observada después de la inmunización experimental o de la exposición natural al parásito no se ha podido establecer aún. El reto para el posible desarrollo de una vacuna basada en la información derivada de la inmunidad adquirida es entender cuáles de las muchas respuestas antígeno-específicas frente a Plasmodium son las predominantes y responsables en la protección.

Para ello, es necesario desarrollar nuevas estrategias metodológicas que permitan identificar antígenos altamente inmunogénicos. En el primer capítulo de este trabajo, hemos desarrollado una metodología que permite la identificación de nuevos antígenos expuestos durante la fase eritrocítica del parásito. Brevemente, en este estudio, se ha optimizado un protocolo de aislamiento y purificación de inmunoglobulinas, mayoritariamente del tipo IgG, que se desarrollan en ratones ICR durante una infección por la cepa letal *Plasmodium yoelii yoelii 17XL*. Un porcentaje elevado de ratones ICR muestran una respuesta inmune humoral eficaz frente a la infección por *Plasmodium yoelii yoelii* 17XL, lo que les permite superar la infección quedando inmunizados frente a reinfecciones. Las inmunoglobulinas IgG presentes en los sueros inmunes de ratón se aislaron y purificaron del resto de proteínas séricas utilizando columnas de afinidad Proteina A/G. Las inmunoglobulinas se inmovilizaron covalentemente en columnas de agarosa que, a su vez, sirvieron para ensayar un protocolo de aislamiento de antígenos de *P. yoelii* capaces de unirse a estos anticuerpos. Los resultados obtenidos indican que las inmunoglobulinas purificadas, de acuerdo al protocolo experimental desarrollado, mantienen su estructura funcional y son útiles para la selección de antígenos reactivos, por lo que pueden ser utilizadas en todos aquellos estudios dirigidos hacia la caracterización de la respuesta inmune humoral frente a la malaria y el desarrollo de nuevas vacunas. Usando espectrometria de masas para analizar las proteínas del parásito aisladas por inmunoafinidad, se identificaron 4 proteínas de *Plasmodiu*: i) protein-disulfuro isomerasa, ii) un miembro

de la familia de proteínas 70 *heat-shock*, iii) plasmepsina y iv) la subinidad de 39 kDa-del factor 3 de iniciación eucariótico. Aunque no se pudieron identificar por espectrometría otros antígenos previamente descritos como dianas en la vacunación frente la malaria, en los geles 1D del eluído tras la purificación de los antígenos sí se observaron bandas que podrían corresponder a la subunidad MSP1-19 y sus precursores.

El segundo capítulo de este estudio se planteó con el objetivo de examinar si los antígenos purificados y aislados mediante inmunoafinidad pueden inducir una inmunidad protectora frente a la fase sanguínea de la infección por *P. yoelii* 17XL. Para ello se realizó un primer ensayo de vacunación comparando las formulaciones de adyuvante CpG ODN_{1826} y Freund´s completo (CFA) e incompleto (IFA) sobre ratones BALB/c, sensibles a la infección por *P. yoelii* 17XL. Utilizando una carga elevada de parásito en la infección en combinación con cantidades limitantes de antígenos, el sistema de Freund´s mostró cierto grado de protección. En un segundo test, utilizando dosis mayores de mezcla de antígenos y tres dosis vacunales, se observó una amplia respuesta inmune frente a extractos totales de *P. yoelii*, analizada mediante Western-blots a diferentes tiempos post-tratamiento en todos los ratones vacunados. A pesar de la evidente respuesta inmune a los antígenos purificados por inmunoafinidad, los ratones vacunados e infectados con dosis letales de *P. yoelii* 17XL mostraron diferentes niveles de protección. Aunque ninguno de los individuos sobrevivió a la infección, el 20% de los ratones sobrevivieron más de 14 días, y el 50% sobrevivió un día más en promedio que los controles, lo que sugiere que se produce cierto grado de protección mediada por la mezcla de antígenos utilizada en el ensayo. En este capítulo se discuten los posibles efectos que pueden tener los distintos antígenos en la protección contra la malaria letal en el modelo de ratón.

Como se ha mencionado anteriormente, la información acumulada sobre estudios de la vacunas en la malaria sugieren que muchas proteínas desconocidas hasta ahora están implicadas en la protección contra la infección. El tercer capítulo de este estudio se ha dirigido a la identificación de antígenos tras la infección con *Plasmodium* en poblaciones de ratones ICR, parcialmente resistentes a malaria, mediante técnicas inmunoquímicas e inmunoproteómicas. Trabajos previos de este

laboratorio han demostrado que, durante la infección con *P. falciparum*, los patrones de oxidación de proteínas, tanto del parásito como del huésped, sufren cambios significativos. Por esta razón, la identificación de las proteínas de parásito carboniladas en ratones infectados puede ser una estrategia valiosa para el descubrimiento adicional de los antígenos aún no identificados y que se puedan utilizar como dianas en nuevas vacunas.

Mediante la comparación de los patrones obtenidos tras transferencia a parejas de membranas de geles 2-D de proteína de *P. yoelii*, la primera revelada con suero obtenido de los ratones ICR infectados y la segunda revelada con anti-2,4-dinitrofenilhidracina (DPNH) tras derivatización con DPNH de las proteínas transferidas, se han podido identificar varias proteínas carboniladas del parásito con actividad inmunogénica en los ratones ICR infectados. Mediante espectrometría de masas de los péptidos generados por digestión con tripsina, las proteínas se identificaron, resultando pertenecer a tres clases: el primero constituido por proteínas de respuesta a estrés, chaperonas y proteínas de invasión (HSP 90, HSP 70, HSP ClpB, co-chaperona GrpE, Cpn 20, protein disulfuro isomerasa, proteína 1 de superficie del merozoito y antígeno de superficie del merozoito PY230), el segundo por proteínas metabólicas (glicerol-3-fosfato deshidrogenasa dependiente de FAD, dihidrolipoamida dehidrogenasa, ATP sintase F1 subunidad beta y adenosina desaminasa), y dos proteínas hipotéticas. Entre todas las proteínas detectadas, ni las proteínas GrpE y Cpn20 ni las cuatro proteínas metabólicas habían sido previamente identificadas como inmunogénicas durante la malaria. En este capítulo se discute la posible importancia de estas proteínas en la infección su potencial como nuevas dianas para vacunas.

CONTENTS

SUMMARY

1 Summary

Despite more than a century of research efforts to eradicate malaria, the disease remains a major, growing threat to the public health and economic development of countries in the tropical and subtropical regions of the world. Human malaria is caused by infection with intracellular parasites of the genus Plasmodium that are transmitted by *Anopheles* mosquitoes. Of the five species of Plasmodium that infect humans, infection with *Plasmodium falciparum (P. falciparum)* is the most lethal. Due to increasing resistance of *P. falciparum* to anti-malarial drugs and insecticides, studies suggest that the number of malaria cases may double in 20 years (WHO, 2007). Thus, the discovery of a vaccine against malaria represents an urgent medical need for the vast population living in areas where malaria is endemic. The ideal vaccine would be cheap, extremely safe, induce life-long immunity, be active against all strains of the parasite and result in nearly complete interruption of the malaria life cycle by vaccine-induced immune responses. While promising results have been obtained, particularly with subunit vaccines, progress toward a malaria vaccine has been slow, owing in part to extensive genetic diversity in candidate vaccine antigens. In subunit vaccination, partial or complete antigens are identified from a pathogen's proteomic complement and used to induce protective immunity to the whole pathogen on vaccination. During the life cycle of *P. falciparum*, several *P. falciparum* polymorphic antigens are exposed to the human immune system. Of these, apical membrane antigen 1 (AMA1), merozoite surface protein (MSP-1) and circumsporozoite protein (CSP) are of particular interest in vaccine development. Epidemiological data suggest that naturally acquired antibodies targeted against merozoite proteins (AMA1 and MSP1) contribute to the acquisition of protective immunity in malaria-endemic areas. Thus, although AMA1 and MSP1 are leading subunit vaccine candidates, their antigenic polymorphism continues to thwart vaccine development.

The human and animal models represented by naturally acquired immunity represent powerful models for the development of a vaccine to completely prevent infection or to prevent death and severe disease, respectively. However, the specific target antigens and epitopes of the protection are largely unknown, and correlates of

protection after experimental immunization or natural exposure are unclear. The challenge for next generation malaria vaccine is to understand which of the many antigen-specific responses might be predominantly responsible for protection, and selection of these antigens is in urgent need for vaccine development.

To achieve this, methodological pathways for the recognition and characterization of highly immunogenic antigens need to be developed. Here, in the first chapter of this work, we have successfully developed a methodology that could permit identification of new antigens exposed during the eritrhocytic stage of the parasite. Briefly, by using specificity IgG-binding domains of recombinant protein A/G, we have isolated a broad range of IgG subclasses from intact serum obtained from ICR mice resistant to the lethal infection by *Plasmodium yoelii yoelii* 17XL. Purified IgGs, were next immobilized covalently onto agarose-loaded spin columns and the assembled columns, were used for isolation of high affinity plasmodial antigens from crude parasite extracts obtained from total parasitized infected RBC (iRBC) from infected mice. By applying mass spectrometry analysis to the parasite proteins isolated by immunoaffinity, we were able to identify 4 plasmodial proteins: i) protein disulfide isomerase, ii) a member of the heat shock protein 70 family, iii) plasmepsin and iv) a 39 kDa-subunit of the eukaryotic translation initiation factor 3. Although we could not identify known vaccine target antigens in plasmodium by MS in our immunoaffinity eluate, the presence of band patterns in the eluate 1D-electrophoregram possibly corresponding to the MSP1 19 kDa subunit and related precursor were observed

The participation of those identified proteins in partial protective immunity in ICR mice population and the new identified ones by their roles to elicit immunity in murine model of malaria, is the main part of this study and are presented in details in chapter one. Interestingly, the procedure of isolation and identification of parasite antigens by using serum IgGs from malaria-protected individuals could be a novel strategy for the development of multi-antigen-based vaccine in human, accordingly.

The second chapter of this study was aimed to examine whether the immunoaffinity-isolated parasite antigens were able to induce protective immunity against blood stage malaria infection. To examine the hypothesis, two adjuvant

formulations were compared in a test vaccine trial: CpG ODN_{1826} system and Freund´s complete (CFA) and incomplete (IFA) system carried out using BALB/c mice strain, susceptible to *P. yoelii* infection. Using a high parasite load on infection, mice treated with limiting amounts of antigen mixture using Freund´s system showed some degree of protection. Hence, in a second vaccination trial, both the amount of total multiple antigens and the number of boosting up were increased to up to 10 µg per each inoculation and 3 times, respectively. Western blot analysis of vaccinated mice serum at different time post vaccination revealed a broad range of reactivity with total plasmodium extracts. Even though all mice have shown strong immune response to multiple affinity purified antigens, different levels of protection in vaccinated mice challenged by a lethal dose of parasite were observed. Although none of the individuals survived the infection, 20% of mice survived more than 14 days and 50% survived one more day compared to controls, suggesting some degree of protection induced by the antigen mixture used in the test. The comparisons of two vaccination trials and analysis of immune response by western blot in each individual vaccinated mouse sera at different time post-vaccination and the possible roles of identified antigens in protection against lethal Plasmodium infection are dicussed in this chapter.

As mentioned above, recently accumulated information on malaria vaccine studies, have shown many unknown proteins are involved in protection against malaria infection. The third chapter of this study, is mainly focused on the identification of antigens in blood-stage Plasmodium infection in ICR mice population resistant to malaria, by using immunochemical and immunoproteomic approaches. Previous work in this laboratory has shown that, upon *P. falciparum* infection of human RBC, both host and parasitic cells suffer significant changes in the pattern of protein oxidation. Hence, the identification of potential carbonylated antigens in the erythrocyte or the parasite in infected mice, may thus be a valuable strategy for the discovery of unknown vaccine antigens. By comparison of 2-D Western blots of total *P. yoelii* protein revealed by using sera from infected ICR mice and, separately, anti-2,4-dinitrophenylhydrazine (DNPH) on membranes derivatized after protein transfer, it was possible the detection of several carbonylated plasmodial proteins. The coincident spots were analyzed by mass spectrometry of triptic digestions, allowing the

identification of several proteins belonging to three different classes: fate proteins (heat shok protein 90, heat shock protein 70, heat shock protein ClpB, co-chaperone GrpE, Cpn 20 protein, protein disulfide isomerase, merozoite surface protein 1 and merozoite surface antigen PY230), metabolic proteins (FAD-dependent glycerol-3-phosphate dehydrogenase, dihydrolipoamide dehydrogenase, ATP synthase F1 subunit beta and adenosine deaminase) and two hypothetical proteins. Interestingly, 2 out of 6 fate protein (co-chaperon GrpE and Cpn20 protein) and all 4 plasmodial metabolic enzymes had not been previously identified as immunogenic proteins during blood-stage malaria infection. The hypothetical roles of the newly identified antigens with those already known potential malaria blood-stage vaccine antigens, are discussed in this chapter.

INTRODUCTION

2 INTRODUCTION

2.1 Malaria in the world

Malaria, an infectious disease caused by several apicomplexa *Plasmodium* species, is an important cause of mortality and morbidity in many regions of the world. Each year, an estimated 300–500 million people are affected worldwide although the true figure may triplicate this number [1]. Malaria kills 1–2 million people each year, mostly children under the age of 5 years and a significant number of pregnant women in sub-Saharan Africa (WHO The World Health Report Geneva 1999). About half the world's population (3.3 billion) live in areas that have some risk of malaria transmission and one fifth (1.2 billion) live in areas with a high risk of malaria (more than 1 reported clinical case per 1000 population per year). Another 2.1 billion live in areas of low risk. Although low-risk areas cover a large number of people living across a wide geographical area, they produce a relatively small number of malaria cases each year (less than 2 million) and account for less than 3% of cases reported by countries in 2006. Africa has the largest number of people living in areas with a high risk of malaria followed by the South-East Asia Region (Figure 1). In recent years, there has also been an increasing number of malaria cases among travelers to endemic countries.

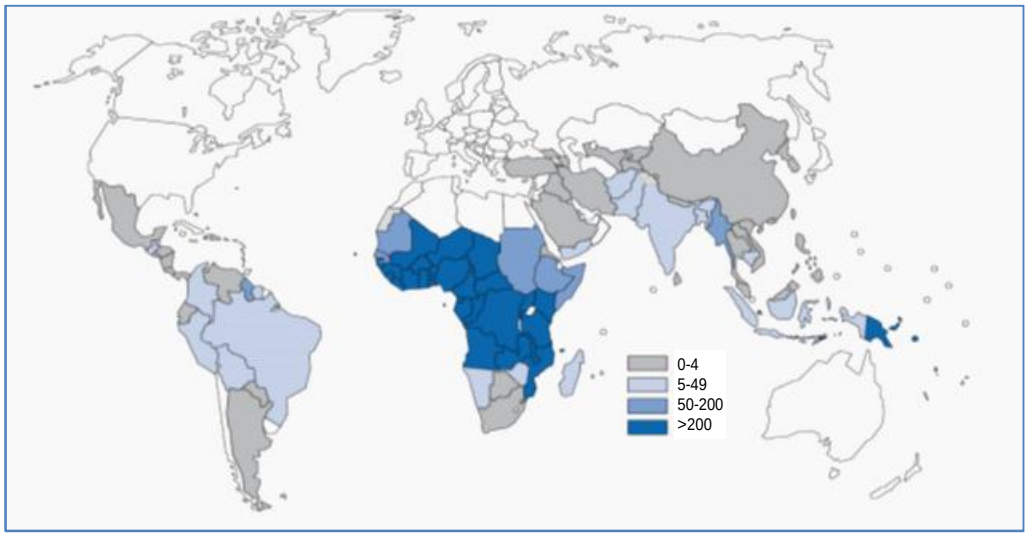

Figure 1. Estimated incidence of clinical malaria per 1000 population. World Malaria Report 2008. http://whqlibdoc.who.int/

2.2 Malaria and the *Plasmodium* life cycle

Malaria is caused by unicellular protozoan parasites of the *Plasmodium* genus. There are four species of malaria parasites that infect humans: *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*. The most severe form of malaria is caused by *P. falciparum.* The severity of the disease depends largely on the species and strain of the infecting parasite, and the immunological status of the infected individual. Cyclical fevers are the hallmark of malaria and typically occur shortly before or at the time of red blood cell (RBC) lysis as schizonts rupture to release new infectious merozoites. This occurs every 48 h in *P. vivax*, *P. ovale* and *P. falciparum*, and every 72 h in *P. malariae* infection Intense fever is accompanied by nausea, headaches and muscular pain, amongst other symptoms. In patients infected with *P. vivax* and *P. ovale*, relapse may recur months to years after initial infection. This is caused by re-activation of the silent liver-stage form of the parasites (hypnozoites)[2].

The disease is transmitted from one infected person to another by the bite of female *Anopheline* mosquitoes. Soon after the female mosquito has ingested blood from an infected person, male gametocytes fertilize female gametocytes. Mobile products of this fertilization, the ookinetes, burrow through the stomach wall and develop into oocysts in the lining of the gut. When the cysts rupture, they release sporozoites which enter the salivary glands. Within 60 minutes of inoculation into a vertebrate host by the mosquito, the sporozoites move to the liver and invade hepatocytes where they remain for 9–16 days and undergo asexual amplification. During this pre-erythrocytic stage, the host is asymptomatic [3]. The erythrocytic stage begins when the infected liver cell bursts, releasing merozoites into the bloodstream. Within 1–2 min of release, each merozoite attaches to specific receptors on the RBC membrane via ligands on the surface of the merozoite. Subsequently, the host RBC membrane invaginates so that the merozoite moves into the erythrocyte. Residing in the parasitophorous vacuole, the parasite undergoes development from the early ring stage trophozoite to the late trophozoite and then, after mitotic divisions, to the schizont stage, which contains 6–32 merozoites, depending on the parasite species [4] . When the erythrocytic schizont ruptures, the merozoites spill into the blood and each one continues the life cycle by invading another RBC. During this repeated cycle, a small proportion of asexual parasites converts to gametocytes that are essential for

transmitting the infection to others through female anopheline mosquitoes, but cause no disease [5]. Then the infectious cycle of *Plasmodium* can repeat itself (Figure 2). It is the asexual blood stage that is responsible for the symptoms of the disease. There is therefore a significant effort to develop a vaccine against this stage of the life cycle, which could limit parasite growth and consequently prevent or minimize clinical disease. The successful development of an asexual blood stage vaccine is critically dependent upon our understanding of immunity to asexual blood stage parasites [2].

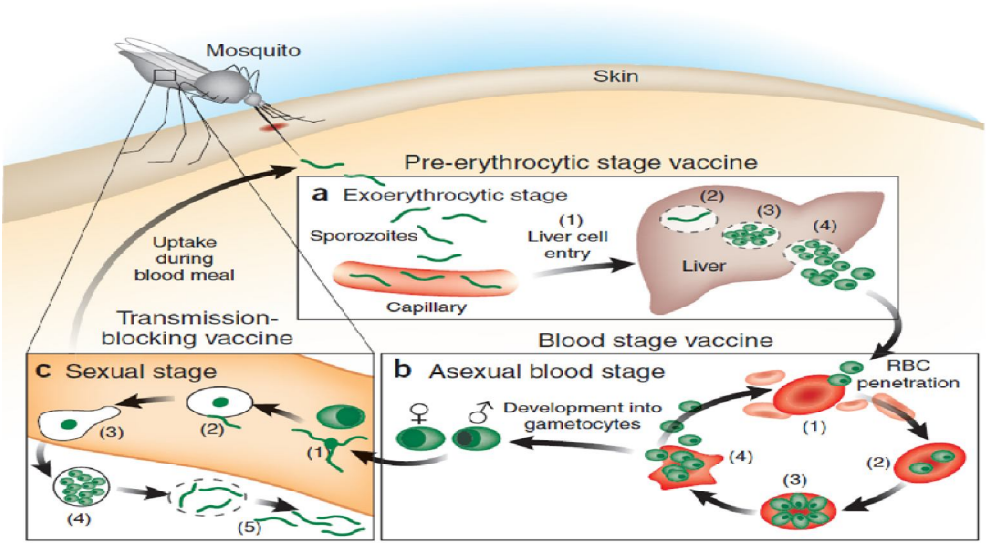

Figure 2. Malaria life cycle of the *Plasmodium* parasite has three distinct stages.Malaria sporozoites are introduced into humans when an infected female *Anopheles* mosquito takes a blood meal. (**a**) During the exoerythrocytic stage, sporozoites make their way through the blood to the liver via Kupffer cells (1). Sporozoites invade numerous hepatocytes (2) and develop into the exoerythrocytic form (EEF), also known as tissue schizonts (3). These EEFs contain many merozoites, which are released and circulate in the blood upon EEF rupture (4). (**b**) The asexual blood stage commences when the merozoite invades a red blood cell (RBC; 1). Within the RBC, the merozoite develops into a trophozoite (2) and then into a schizont (3). The asexual blood stage cyclically continues as schizonts rupture, releasing merozoites for further invasion of RBCs (4 and 1). Male and female gametocytes are also produced during this stage and are taken up by the mosquito when feeding (see panel **c**). (**c**) The sexual stage takes place within the gut of the mosquito. Inside the gut, the gametocytes (1) are released and fuse to form a zygote (2), which in turn develops into an ookinete (3). The ookinete crosses the midgut epithelial cell wall. Between the epithelial cell wall and the basal lamina of the midgut, the ookinete develops into an oocyst (4). The oocyst produces thousands of sporozoites, which are released into the hemocele (the body cavity) and are carried in the hemolymph to the salivary glands (5), ready for injection into the next host. (Adapted from [6])

2.3 Plasmodium parasites and mice models

Four species of murine parasites are available to model human malaria: *P. chabaudi, P. berghei, Plasmodium vinckei* and *P. yoelii*. Among them, *P. chabaudi* provides an excellent experimental tool with many similarities to *P. falciparum*. The sequencing of the genomes of both *P.falciparum* and *P.chabaudi* is facilitating comparative analysis of antigens that may be important in understanding the immunobiology of malaria, as well as offering novel candidate vaccine targets [7]. These include analogous blood-stage antigens, invasion of immature and mature erythrocytes, suppression of B- and T-cell responses, and parasite sequestration in liver and spleen [8]. In this infection model, a rapid multiplication of the parasite during the first week post infection (*pi*) is followed by a curative phase in resistant mice with immune and erythropoietic responses, eliminating the parasites by the fourth week *pi*. Susceptible animals lack these responses and usually die during the second week *pi*. Two subspecies of *P. chabaudi* have been defined: *P. chabaudi chabaudi* and *P. chabaudi adami*. Both have been extensively used to study the immunological basis of some of the pathologies of human malaria and in genetic linkage studies to map loci controlling parasitic levels. *P. berghei* is a useful experimental model of cerebral malaria. *P. yoelii* is the third most common parasite in mouse malaria studies. There are three recognized subspecies: *P. yoelii yoelii*, *P.yoelii killicki* and *P. yoelii nigeriensis*. Most of them have been widely used as models for the development and characterization of vaccine candidates, in the investigation of red blood cell invasion and, more recently, in genetic linkage studies. The least studied of the parasite *P. vinckei* is, however, the most widely distributed of the murine species. Four subspecies are recognized: *P. vinckei vinckei*, *P. vinckei petteri*, *P. vinckei lentum* and *P. vinckei brucechwatti*. *P. vinckei vinckei* and *P. vinckei petteri* have been used in several chemotherapeutical studies and in the identification of new antimalarial drug targets [8].

Table 1. Plasmodium infections in different strains of mice. BALB/c mice can be susceptible or resistant to Experimental Cerebral Malaria (ECM) caused by *P.berghei* ANKA depending on the sourse. *L* letal, *NL* non-lethal. (Adapted from [9].

Parasite	Strain	Mouse strain	Lethality	Experimental use
P. chabaudi chabaudi		CBA C57BL/6 BALB/C	NL	Immune mechanisms Malaria associated clinical signs/sequestration
P. chabaudi chabaudi	AS	129sv A/J DBA/2	L	Pathogenesis Chemotherapy Resistance and susceptibility Immune mechanisms
P. chabaudi chabaudi	CB	CBA C57BL/6	L	Pathogenesis Chemotherapy Resistance and susceptibility Immune mechanisms
P. chabaudi adami		BALB/c C57BL/6	NL	Immune mechanisms
P. berghei	ANKA	BALB/c C57BL/6 CBA	L	Pathogenesis ECM/sequestration
P. berghei	K173	BALB/c CBA DBA C57BL/6	L	Pathogenesis Control for ECM
P. yoelii	17XL	CBA BALB/c C57BL/6 DBA Swiss	L L	Immune mechanisms Pathogenesis ECM/sequestration
P. yoelii	YM	CBA BALB/c C57BL/6 DBA	L	Vaccine Pathogenesis Hypoglycaemia
P. yoelii	17XNL	CBA BALB/C C57BL/6 DBA	NL	Immune response Vaccination Immune mechanisms
P. vinckei vinckei		BALB/c	L	Chemotherapy Pathogenesis Malaria associated clinical sign/sequestration
P. vinkei petteri	CR	C57BL/6 BALB/c	NL	Immune mechanisms

2.4 Malaria and the immune response

In populations continuously exposed to malaria, several types or levels of immunity against *P. falciparum* co-exist. Immunity to severe clinical episodes (including cerebral malaria, severe anaemia, metabolic acidosis, and other severe manifestations)

is observed in older children and adults, is acquired relatively early (usually complete by the age of five years), and lasts for life [10, 11]. Immunity to mild clinical episodes takes longer to establish. Young adults remain susceptible often until their middle to late twenties. Sterile immunity to infection with parasites is rarely, if ever, observed. The prevalence of *P. falciparum* parasitaemia increases steeply in early childhood, remaining high for a period of time after, immunity to severe and mild disease is well established. Thus, the expression of the acquisition of immunity appears to be sequential, with the ability to limit parasite growth and multiplication, followed by essentially complete protection against severe and then mild clinical disease, and culminating with partial protection against infection. Both innate [12] and acquired immunity [13] appear to take place in human and in rodent models, involving antibody and cell-mediated processes.

2.4.1 Antibody-mediated immunity to asexual blood stage malaria

It is well established that B cells and Ab play a crucial role in immunity to malaria. It has been demonstrated that naturally acquired immunity to malaria in individuals living in endemic areas, which takes several years to develop, is thought to be largely dependent largely on the acquisition of a repertoire of specific, protective Ab directed against several polymorphic target antigens. The unequivocal identification of such antigens has not been, however, feasible. The best protective antigen so far proposed, *P. falciparum* erythrocyte membrane protein-1 PfEMP-1 [14], has been associated with protection in some studies, but not in others [15-23]. Treatment of *P. falciparum* infected Thai patients with IgG extracted from immune African adults resulted in reduction of parasite load and clinical symptoms [24]. In mice, passive transfer of monoclonal Ab (mAb) against parasite antigens confers protection in naive mice [25, 26]. Immunoglobulin (Ig) μ-chain gene-targeted (μ-MT) mice lacking B cells are unable to clear parasites from *P. chabaudi chabaudi* AS infection, and instead develop chronic parasitaemia [27, 28]. Infected μ-MT mice treated with antimalarial drugs develop acute parasitaemia during secondary infection that resembles a primary infection, although with a reduced parasite density [28] and the subsequent chronic infection is not resolved, indicating that B cells are required for

development of protective immunity. Adoptive transfer of B cells from immune donors at a later stage of infection restores the ability of deficient mice to complete parasite clearance, confirming the critical role of B cells in the clearance of blood stage parasites [28]. Studies in B-cell deficient mice demonstrate that B cells also play an essential role in switching from a Th1 response to a Th2 response, which is critical for the complete resolution of *P. chabaudi* infection [27]. The degree of protective immunity in humans [29], monkeys [30] and mice [31, 32] has been shown to correlate with the level of Ab against asexual blood stage antigens, and is dependent on Ab isotypes. The IgG subclass responses against ring-infected erythrocyte surface antigen (RESA), merozoite surface protein (MSP) 1, MSP-2 and crude [33] *P. falciparum* antigen in people living in exposed areas are partly determined by host genetic factors and are age dependent. Cytophilic Ab of the IgG1 and IgG3 subclasses are considered to be the most important Ab for protection against *P. falciparum* malaria in humans [33]. Acting in collaboration with effector cells such as monocytes and macrophages, they mediate opsonization and Ab-dependent cellular inhibition. A seroepidemiological study has shown that increased levels of *P. falciparum*-specific IgG1 and IgG3 in individuals living in endemic areas are associated with lower parasitaemia and reduced risk of malaria pathology [34, 35].

Protection attributable to parasite-specific IgG3 is age-associated, with greater levels of protection seen in adults. In addition to IgG1 and IgG3, IgG2 may be involved in protection. High levels of IgG2 to RESA and to MSP2 are associated with resistance to *P. falciparum* at the end of the transmission season and levels tend to be higher in older individuals who are better protected against infection and disease [36]. In contrast, levels of IgG4 to parasite extract, RESA, MSP1 and MSP2 are lower in individuals who do not develop malaria than in susceptible individuals and are positively correlated with risk of infection. It has been suggested that IgG4 competes with cytophilic Ab for antigen recognition and may therefore block cytotoxicity mediated by Ab activated effector cells [36].

From a mouse model, Jayawardena *et al*. [37, 38] demonstrated that the passive transfer of hyperimmune serum to CBA mice completely protected them against challenged with 10^4 *P. yoelii* 17XNL-parasitized erythrocytes. In addition, passively transferred antibodies (Ab) delayed the onset of infection when larger

inocula (5×10^4 or 1×10^5 PRBC) were used [38]. Similar results have been reported for BALB/c mice [39]. A heterogenous Ab response is induced by *P. yoelii*. Langhorne et al. investigated the antimalarial Ab response to *P. yoelii* 17XNL in C3H mice and found that immunoglobulin M (IgM), IgG1, IgG2, and IgG3 Ab were produced, with the highest titers being of the IgG2 isotype [40].

In mice, the cytophilic isotype, IgG2a, is associated with protection against *Plasmodium* infection [41, 42]. IgG2a is predominant during the primary ascending parasitaemia in mice infected with *P. chabaudi* AS followed by an IgG1 response during the chronic stage of infection, as a consequence of Th1 to Th2 switching [43]. It has been shown that IgG1 and IgG2b can confer protection against lethal challenge infection with *P. yoelii* YM in mice immunized with $MSP1_{19}$ [31]. IgG3 may also be important, as passive transfer of anti-$MSP1_{19}$ IgG3 into naive recipients resolves *P. yoelii* infection [44, 45]. Antibody responses directed against surface proteins of the merozoite may function either by blocking RBC invasion or by making the merozoite susceptible to phagocytosis. Parasite antigen-specific Ab play an important role in controlling parasitaemia via Ab-dependent cellular inhibition (ADCI), whereby binding of antibodies to phagocytes via Fc receptors leads to inhibition of parasite growth [24, 36, 46, 47]. It has been demonstrated that specific Ab initiate parasite clearance by opsonization, thus enhancing the activity of phagocytic cells [48] or initiating complement-mediated damage [49, 50].

Despite the importance of Ab responses for protection against malaria, it seems that not all Ab are protective. Polyclonal Ab specific to MSP2, but not mAb specific to the same antigen, enhance invasion of multiple merozoites into RBC [51, 52]. Furthermore, these MSP2-specific Ab at high-titre fail to induce complement-mediated damage. In another example, mAb against $MSP1_{19}$ which inhibit RBC invasion by merozoites and prevent MSP-1 secondary processing, can be blocked by other mAb to the same antigen [53]. These studies illustrate the importance of identifying epitopes that induce protective Ab when designing a vaccine against malaria.

2.4.2 Cell-mediated immunity and malaria

CD4 T cells are classified into two major subsets according to their pattern of cytokine production. Th1 cells produce interleukin (IL)-2, interferon (IFN) γ, and tumour necrosis factor, whereas Th2 cells produce IL-4, IL-5, IL-6 and IL-10 [54]. In general, Th1 cells are responsible for cell-mediated immunity (CMI). They activate macrophages and other cells to produce mediators through the release of inflammatory cytokines. In Th1 cytokines, IFN γ has been shown to be associated with clearance and protective immunity in malaria [55-61]. Th1 cells enhance the production of Ab that promote opsonization and phagocytosis, mainly IgG2a and IgG3 in mice, and IgG1 and IgG3 in humans. Th2 cells regulate humoral immunity by providing help to B cells for the production of Ab. Th2 cells promote the production of IgG subtypes that are associated with allergies and helminthic infections, such as IgG1 in mice and IgG4 in humans. However, considerably less is known about the Th2 response and the role of Th2 cytokines in malaria disease.

2.4.3 Mediators of cell-mediated immunity to malaria

Interleukin-12 is a key cytokine that initiates Th1 responses by triggering IFNγ production from natural killer (NK) and CD4 T cells [62]. Interleukin-12 secretion is induced by various infectious agents, including viruses, bacteria and parasites. During malaria infection, early non-specific immune responses can be augmented by the release of IL-12 from splenic macrophages [63, 64]. Administration of anti IL-12 Ab to normal mice during *P. berghei* infection results in a marked reduction of IFNγ production, showing that IL-12 is a potent inducer of IFNγ during malaria infection [65]. Treatment of *P. chabaudi* AS susceptible A/J mice with IL-12 results in increased numbers of NK cells which spontaneously secrete IFNγ and TNFα [66]. Consequently, IL-12 treated A/J mice are able to eliminate parasites and survive infection, whereas untreated A/J mice develop high parasitaemia and die. The ability of mice to control parasite growth is abrogated when the mice are depleted of NK cells, indicating that the protective effects of IL-12 are mediated by NK cells [66, 67]. In addition to activation of NK cells, IL-12 enhances production of IFNγ by CD4 T cells, which is also critical for protection [68]. Taken together, the data indicate that IL-12 plays an

important role in protective immunity to blood stage malaria by inducing IFNγ production by NK and CD4 T cells. In the mouse model, infection of IFNγ-deficient mice with *P. c. chabaudi* AS results in increased morbidity and mortality, [69, 70] indicating a role for this cytokine in protection.

Recruitment and local proliferation of macrophages are also impaired in the absence of IFNγ [69]. Mice defective in IFNγ and its receptor show a predominantly Th2 response, which is associated with susceptibility to *P. chabaudi* infection [69, 71]. Thus, IFNγ is critical for resistance to blood stage malaria, through the stimulation of cytokine production by effector cells and enhanced activity of macrophages. Tumour necrosis factor α production is greatly augmented during malaria infection, as shown by elevated levels of TNFα in plasma of patients with malaria [72, 73] and infected mice [67, 74]. As well as being induced by cytokines such as IFNγ, [69] TNFα release by macrophages can also be directly induced by malaria parasites and their soluble antigens, such as malaria pigment (haemozoin) [75] and glycosylphosphatidylinositols [76]. High levels of TNFα mRNA expression in the spleens of C57BL/6 mice correlate with resistance to *P. chabaudi* AS infection, and administration of anti-TNFα Ab to resistant mice abrogates the immunity, indicating a protective role for TNFα [77]. Mouse sera containing TNFα inhibit growth of *P. falciparum in vitro* by causing deterioration and degradation of parasites, suggesting that TNFα has a non-specific inhibitory effect on the parasites [78, 79]. Collectively, the data demonstrate that cell-mediated immunity can be beneficial or detrimental to the malaria infected host. To achieve desirable outcomes, the balance of mediators that are involved in the immune response to malaria must be tightly controlled. An understanding of the mechanisms by which cytokines induce protection and/or pathology in malaria will prove to be fundamental for designing vaccines and developing new therapies for malaria.

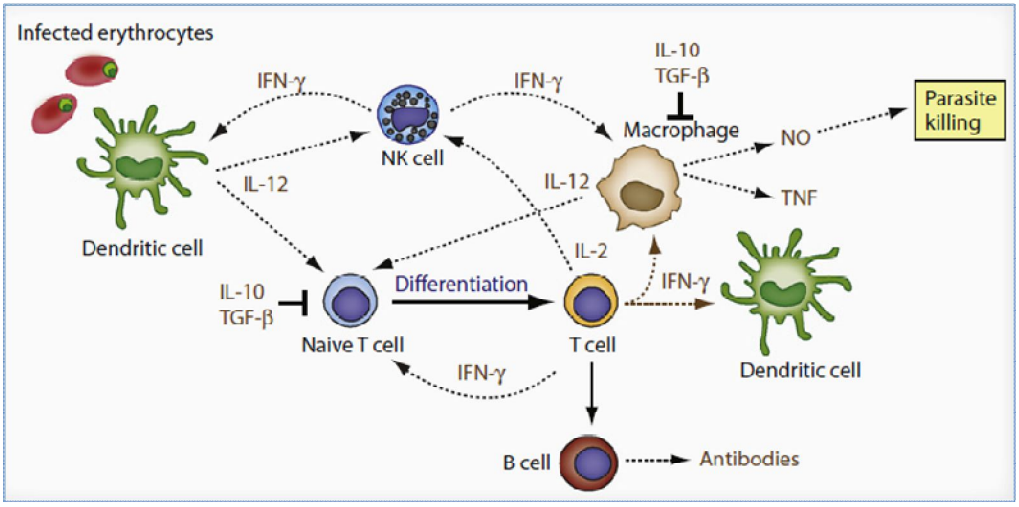

Figure 3. Linking Innate and Adaptive Immunity to Blood-Stage Malaria Possible regulation of adaptive immunity to bloodstage malaria by cytokines produced by cells of the innate immune response. In response to parasite ligands recognized by pattern-recognition receptors (PRRs), such as Toll-like receptors (TLRs) and CD36, or inflammatory cytokines, such as interferon-γ (IFN-γ), dendritic cells (DCs) mature and migrate to the spleen—the primary site of immune responses against blood-stage Plasmodium parasites. Maturation of DCs is associated with the upregulation of expression of MHC class II molecules, CD40, CD80, CD86, and adhesion molecules and the production of cytokines including interleukin-12. IL-12 activates natural killer (NK) cells to produce IFN-γ and induces the differentiation of Th1 cells. The production of cytokines, particularly IFN-γ, by NK cells results in DC maturation and enhances the effect of parasite-derived maturation stimuli, facilitating the clonal expansion of antigen-specific naive CD4+ T cells. IL-2 produced by antigen-specific Th1 cells further activates NK cells to produce IFNγ, which induces DC maturation and activates macrophages, further amplifying the adaptive immune response. Cytokines such as IL-10 and transforming growth factor-β (TGF-β) negatively regulate both innate and adaptive responses. NO, nitric oxide; TCR, T cell receptor; TNF, tumor-necrosis factor. (Adapted from [80]).

2.5 Selection of antigens for blood stage malaria vaccine

Since clinical symptoms of malaria manifest only during the blood stage, a vaccine against this stage of the parasite life cycle would prevent or reduce severity and complications of the disease, and perhaps eliminate malaria if sterile immunity could be achieved. In red cells, parasites appear well located to avoid host responses. Red cells lack class I and class II MHC molecules and antigen-processing machinery. Therefore, direct T cell-mediated responses are not induced to determinants presented at the host cell surface. Only at schizont rupture is the parasite directly exposed, when, for a very brief period, daughter merozoites have to attach to and enter new red cells. Much attention has therefore been given to parasite molecules that interact with the host cells during RBC invasion as potential targets of host immune responses. A number of proteins have been identified on the merozoite

surface or in the apical organelles that play a role in RBC invasion and are thought to be targets of immunity.

1.5.1 Merozoite surface proteins

Among these molecules merozoite surface proteins are potential malaria vaccine candidates as they play important roles in the initial recognition and attachment of merozoites to the RBC surface [81-84]. As proteins on the merozoite surface are exposed to the host immune system, they are thought to be targets of immune response [85, 86]. Merozoite surface protein 1 is a glycoprotein synthesized as a high molecular weight (~185–205 kDa) precursor protein [87-89]. The protein precursor is processed at least twice by protease enzymes into a number of fragments. At schizont rupture, primary processing occurs, giving rise to major fragments of approximately 83 ($MSP1_{83}$), 28–30 ($MSP1_{30}$), 38 ($MSP1_{38}$) and 42 ($MSP1_{42}$) kDa found as a non-covalently associated complex held together on the free merozoite surface by the 42 kDa fragment [87-89] (Figure 4). At the time of merozoite invasion, secondary processing, which is a prerequisite for RBC invasion, takes place [90].

Epidemiological studies in malaria endemic areas show that PfMSP-1_{19} seropositive children are significantly more resistant to clinical malaria than seronegative children [18, 19]. In the same study, human antibodies to PfMSP-1_{19}, acquired in response to natural infection, inhibited merozoite invasion of erythrocytes. Since this study was conducted using affinity-purified antibodies, inhibition did not require the presence of either complement or mononuclear cells, occurred at physiological antibody concentrations and was equally effective against parasites expressing both major sequence variants of PfMSP-1_{19} [91].

However the epidemiological evidence of the protective effect of naturally acquired anti-merozoite responses has not been clearly stablished in malaria endemic populations [22]. In a recent study conducting a Meta-analysis Of Observational Studies in Spidemiology (MOOSE), some support for the protective effect of total IgG responses to particular merozoite surface antigens against symptomatic *P.falciparum* malaria has been found in human asindividuals having IgG against MSP-3-Ct and MSP-1_{19} appeared to have a lower risk (54% and 18%, respectively) of symptomatic *P. falciparum* infection as compared to individuals . that iswithout detectable IgG.[92]

The whole molecule of MSP1 has been shown to induce protection against lethal *P. yoelii* YM and *P. falciparum* in mice and monkeys, respectively [93, 94].

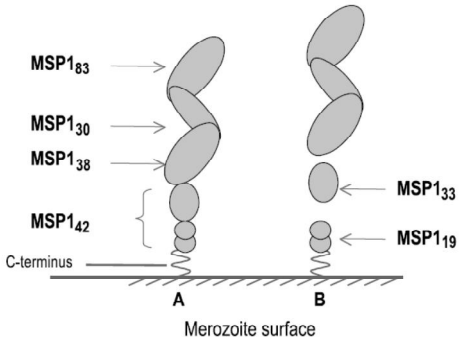

Figure 4. Schematic showing processing of merozoite surface protein 1 (MSP1)(modified from Blackman *et al.* [95]) Panel (A) shows primary processing, and (B) shows secondary processing. (Adapted from [2]).

Immunization of mice with the C-terminal proteins, MSP1$_{19}$ and MSP1$_{42}$, induces protection against *P. yoelii* infection [96] and passive transfer of mAb specific to MSP1$_{19}$ or MSP1$_{42}$ suppresses parasitaemia [26], indicating that MSP1$_{19}$ and MSP1$_{42}$ also play an important role in protective immunity.

1.5.2 Apical Merozoite surface antigen 1

Apical merozoite surface antigen 1 (AMA1) is an integral membrane protein located in the apical secretory organelles or rhoptries, of developing and free merozoites. AMA1 has been found in all Plasmodium species studied and is a target of antibodies that neutralize invasion of erythrocytes [97, 98]. Protective immune responses induced by AMA1 have been shown in mice [99, 100] and monkeys [101, 102]. Although the specific function of AMA1 during merozoite invasion is unknown, PyAMA1 has been identified as an erythrocyte binding protein, and MAb 45B1 against PyAMA1 blocks this ligand-receptor interaction [103]. Native PyAMA1 induces a protective response, and passive immunization with MAb 45B1 protects against a lethal blood-stage infection with the YM line of *P.yoelii* [25].

1.5.3 High-molecular-mass rhoptry proteins

Another target of antibodies that protect against the virulent YM parasite is the family of high-molecular-mass rhoptry proteins (PyP235) [93, 104]. PyP235 is coded by a multigene family [105, 106], and at least one of the PyP235 proteins binds to the surface of mouse erythrocytes [107, 108], suggesting that they are involved in erythrocyte recognition and invasion. PyP235 proteins are members of a superfamily that includes reticulocyte binding proteins of *P. vivax* [109] and proteins recently identified in *P. falciparum* [110-112]. Immunization with PyP235, or passive immunization with two MAbs (25.77 and 25.37) directed against PyP235, protects BALB/c mice against challenge with the virulent *P.yoelii* YM line [93, 104], restricting the parasite to reticulocytes. PyP235 binds preferentially to mature erythrocytes, and the binding is inhibited by specific antibodies, suggesting a role in the invasion of mature erythrocytes but not reticulocytes [113]. Distinct subsets of Py235 are expressed in sporozoite, hepatic merozoites and erythrocytic merozoites [114] indicating a conserved role of Py235 in the invasion of different host cell populations.

2.6 Malaria vaccines

Although an increased number of tools for the control of malaria are now available, the majority of these are at best, partially effective and require substantial training and resources to implement at national levels [115]. Many malaria control experts argue that effective vaccination against malaria is in fact, the only realistic long-term solution for resource-poor countries [116]. To date, in spite of many years of dedicated and high quality research, there is still no effective malaria vaccine, although the continous progress makes reasonable to expect some succes in the short-to medium term. Most efforts to develop a malaria vaccine are focused on *P. falciparum* as it is responsible the most severe forms of the disease. The belief that it will be possible to develop an effective vaccine for malaria stems from three principal observations. First, complete protection against experimental sporozoite challenge has been achieved in rodents [117], non-human primates [118] and humans [119, 120]. Second, individuals living in endemic areas naturally acquire non-sterile immunity to malaria in which they are protected from severe illness and death, while remaining

susceptible to infection [10]. Third, in classic experiments, passively transferred antibodies from malaria 'immune' individuals were effectively used to treat both children and adults with severe malaria [121, 122]. Two main strategies for malaria vaccine development are currently being pursued, largely in parallel: the sub-unit approach and the whole-organism approach.

1.6.1. Sub-unit Vaccines

The rationale behind these vaccines includes the ability to block the molecular interactions (eg. receptor-ligand binding) that are known to occur between the host and parasite, for example, during invasion of hepatocytes and erythrocytes by sporozoites and merozoites, respectively. It also includes the ability of single antigens to induce protective cell- or antibody-mediated immune responses. For example, antibodies that block the binding of merozoite surface proteins which mediate invasion of red blood cells could halt the proliferation of blood stage parasites, thereby preventing disease. Sub-unit vaccines can be made up of single or multiple antigens, and can target a single stage of parasite development or target multiple stages. Alternatively, they can be designed as multi-epitope vaccines, containing a string of immuno-dominant epitopes from a combination of antigens. Stage-specific vaccines can be classified as pre-erythrocytic, erythrocytic and sexual-stage based on the antigens they contain.

Of all the sub-unit pre-erythrocytic vaccine candidates currently under development, the one based on the CSP has shown some encouraging results. In the RTS,S/AS02 vaccine, the central tandem (asparagine-alanine-asparagine-proline, NANP) repeat and carboxy-terminal regions of CSP are fused to the S antigen of hepatitis B virus (HBsAg) and co-expressed in yeast with un-fused HBsAg. The resulting complex is formulated with the adjuvant AS02 (GlaxoSmithKline Biologicals) which contains an oil-in-water emulsion and immuno-stimulants. In phase IIa trials, RTS,S had a protective efficacy of 41% (95% confidence interval (CI) 22-56%, p = 0.0006) against experimental sporozoite challenge of malaria-naïve volunteers [123]. In a phase IIb trial in Gambian adults, it had a modest protective efficacy against time to first

infection of 34% (95% CI 8-53%), though this protection appeared to be short-lived [124]. In African children however, the results of both phase I and IIb trials have been incrementally encouraging. In this population, RTS,S has been shown to be safe, well-tolerated and immunogenic both in older children aged 1-4 years [125-128] and in infants [129]. Although its reported vaccine efficacy against clinical disease has been modest (30-35%), its protective efficacy against severe disease has been substantial and sustained, 58% (95% CI 16-81%) at 6 months, 49% (95% CI 12-71%) at 18 months [125, 130].

Some are skeptical about RTS,S, in part because its precise mechanism of action remains unclear [131, 132]. While there is evidence that it induces high levels of IFN-γ producing CD8+ (non-cytolytic) and CD4+ T cells, as well as antibodies, particularly in the presence of key adjuvants, these immune responses have not consistently correlated with protection [133, 134]. Others have shown using transgenic parasites that sterile protection against malaria can be obtained independently of immune responses to CSP. Mice immunized with irradiated wild type *P. berghei* were completely protected against challenge with parasites in which *P. berghei* CSP had been replaced with that of P. falciparum, a result which could not be accounted for by cross-reactivity of responses to CSP [135]. From a separate viewpoint, unlike other malaria vaccine candidates, there is little evidence that immune pressure from the human host has driven the polymorphisms observed in CSP in natural infections [136, 137], neither is there evidence that RTS,S-induced immune responses select for parasites bearing divergent CSP alleles (as opposed to those contained in the vaccine) as might have been anticipated [138].

With regard to erythrocytic stage vaccines, the furthest along the line of vaccine development are based on MSP-1, -2, -3, apical membrane antigen 1 (AMA-1) and glutamate rich protein (GLURP) [139]. The first field trial of an asexual blood-stage vaccine was that of the Combination B vaccine, which contains MSP-1 (a 175 amino acid fragment from the relatively conserved blocks 3 and 4 of the K1 parasite line), MSP-2 (the 3D7 allelic type, nearly full length protein), CSP (a T cell epitope), and the ring-infected erythrocyte surface antigen (RESA, containing 70% of the native protein from the C terminal end of the molecule). In a phase 1-2b trial, 120 children in Papua

New Guinea were randomized to receive either vaccine or placebo. The 60 children in each arm were further randomized into two equal groups, one with pre-treatment with sulphadoxine-pyrimethamine (SP) to clear parasites at the start of the study (n= 30), and the other to no-SP treatment (n=30). Although this vaccine had no effect on clinical disease, parasite densities were lower in the vaccine group compared to the placebo group (vaccine efficacy 62% (95% CI 13 – 84)), but only in children who were not pre-treated with SP (n=30). Based on these results, the authors concluded that the MSP-2 component of the vaccine had a specific effect on parasite growth and multiplication, and moreover had induced selection pressure on the parasites [140]. In the same study, high 3D7-specific antibody titres were obtained post vaccination in both the SP and No-SP pre-treatment groups, casting doubt on the mechanism by which vaccine-induced antibodies reduced parasite densities only in the latter group [141]. Although widely quoted, this combination B vaccine study provides remarkably weak evidence in support of arguments to include the major allelic types of antigens in blood-stage malaria vaccines.

In contrast to the pre-erythrocytic and erythrocytic stages, sexual-stage vaccines aim to block malaria transmission from infected hosts, thereby providing a herd benefit for future exposed populations. Studies on two leading candidates for malaria transmission blocking vaccines in humans have been conducted for Pfs25 and Pvs25 proteins expressed on the surface of ookinetes in the mosquito stage of *P.falciparum* and *P.vivax*, respectively [142], where vaccine induced antibodies to Pvs25 (a protein found on the surface of ookinetes) induced significant transmission blocking activity as detected by the membrane feeding assay [143].

Vaccines combining several key antigens from multiple stages would have the potential to interrupt the life cycle of the parasite at multiple points. In a multi-antigen, multi-stage vaccine for instance, parasites that survived the range of immune responses induced by pre-erythocytic antigens, would then have to contend with those induced by blood stage antigens. The different antigens could be selected to target discrete pathways essential to parasite survival within each stage, and ideally induce both antibody- and cell-mediated immunity. In addition, the antigens could be sufficiently diverse to overcome the genetic restriction of the host immune response

and polymorphism of critical target epitopes. While attractive conceptually, few human trials with multi-stage, multi-component vaccines have actually been performed. The first synthetic malaria vaccine (SPf66) to be tested in malaria-endemic areas contained multiple components from both the pre-erythrocytic and erythrocytic stages of P. falciparum [144]. Despite initial promise, a recent meta-analysis of ten trials conducted in malaria-endemic areas found no evidence of protection conferred by vaccination with SPf66 [145]. Of the many lessons that could be learned from the 'failure' of Spf66 [146], perhaps the most important is the need to understand the mechanism(s) by which future vaccines exert their anti-malarial effects. Improvements to Spf66 were precluded in part, by a lack of understanding of its mode of action [147]. Vaccine induced total IgG antibodies to Spf66 did not correlate with the ability of sera to inhibit growth, or with partial clinical protection [148]. In a separate attempt, vaccination with NYVAC-Pf7, a pox-vectored malaria vaccine that contained seven antigens from all three stages of the parasite life cycle within the human host (CSP, PfSSP2, LSA1, SERA, AMA1, Pfs25), also yielded disappointing results when volunteers were challenged [149].

1.6.2. Whole-organism vaccines

The whole organism vaccine approach shares the same foundations as the pre-erythrocytic vaccines: the demonstration of sterile immunity following immunization with radiation-attenuated sporozoites. Volunteers were infected with *P. falciparum* and were treated with doses of chloroquine sufficient to suppress but not eradicate the parasites. Gametocytes were allowed to develop and then mosquitoes were fed on the volunteers [119]. This approach was simplified with the advent of methods to culture *P. falciparum* in vitro [150], produce gametocytes in culture (Campbell, Collins et al. 1982) and infect mosquitoes from in vitro gametocyte cultures [151]. More recent studies have confirmed that the method works in principle, but requires 1000 or more infective mosquito bites, making it logistically impractical to implement on a large-scale [120].

An alternative strategy for the whole organism approach is the use of genetically-attenuated parasites. In place of radiation, genetic engineering is used to attenuate sporozoites such that they remain viable, are able to infect hepatocytes, but unable to develop into mature pre-erythrocytic forms. Importantly, this can be reproduced consistently and in a standardized fashion. In a mouse model infected with *P. berghei*, inactivation of liver-stage specific genes, UIS3 and UIS4 (up-regulated in infective sporozoites) or the sporozoite-specific gene P36p, render parasites incapable of completing their intra-hepatic development, but induce immune responses that confer sterile protection when challenged with wild type infectious sporozoites [152, 153].

2.7 Malaria and vaccine adjuvants

Adjuvants are molecules, compounds or macromolecular complexes that boost the potency and longevity of specific immune response to antigens, but cause minimal toxicity or long lasting immune effects on their own [154]. The addition of adjuvants to vaccine enhances, sustains and directs the immunogenicity of antigens, effectively modulating appropriate immune responses, reducing the amount of antigen or number of immunization required and improving the efficacy of vaccines in newborns, elderly or immune-compromised individuals [155].

It is generally accepted that subunit vaccines for malaria will require adjuvants to induce protective immune responses, and availability of suitable adjuvants has in the past been a barrier to the development of malaria vaccines. Several novel adjuvants are now in licensed products or in late stage clinical development, while several others are in the earlier development pipline [156]. Successful vaccine development requires knowing which adjuvants to use and knowing how to formulate adjuvants and antigens to achieve stable, safe and immunogenic vaccines [156].

Adjuvants can be classified according to their component sources, physiochemical properties or mechanisms of action [156, 157]. Two classes of adjuvants commonly found in modern vaccines include:

i) Immunostimulants (Table 2) that directly act on the immune system to increase responses to antigens. Examples include: TRL ligands, cytokine, saponins and bacterial exotoxine that stimulate immune responses.

Table 2. Immune response triggered by immunostimulants. (Adapted from [157]).

Immunostimulant	Cellular interaction	Type of immune response
TRL ligands		
Bacterial lipopeptide, lipoprotein and lipoteichoic acid; mycobacterial lipoglycan; yeast zymosan, porin	TLR-2, 1/2 , 2/6	Th1,antibody (Ab), NK cell
Viral double stranded RNA	TLR-3	NK cell
Lipopolysaccharide, Lipid A, monophosphoryl lipid A (MPL®), AGPs	TLR-4	Strong Th1,Ab
Flagellin	TLR-5	Th1, CTL, Ab
Viral single stranded RNA, imidazoquinolines	TLR-7/8	Strong Th1, CTL
Bacterial DNA, CpG DNA, hemozoin	TLR-9	Strong Th1, CTL and Ab; NK cell
Uropathogenic bacteria, protozoan profilin	TLR-11	Th1
Others		
Saponins (Quil-A, QS-21, Tomatine, ISCOM, ISCOMATRIX™)	Antigen processing	Strong Th1, CTL and Ab; long term memory
Cytokines: GM-CSF, IL-2, INF-γ, Flt-3.	Cytokine receptors	Th1, Ab
Bacterial toxins (CT, LT)	ADP ribosylating factors	Ab

ii) Vehicles (Table 3) that present vaccine antigens to the immune system in an optimal manner, including controlled release and depot delivery systems to increase the specific immune response to the antigen. The vehicle can also serve to deliver the immunostimulants described in the previous point. Examples include: mineral salts, emulsions, liposomes, virosomes (nanoparticles made of viral proteins such as influenza hemagglutinin and phospholipids), biodegradable polymer microspheres and so-called immune stimulating complex.

Table 3. Immune response triggered by vehicle or delivery system. (Adapted from [157]).

Vehicle or delivery systems	Type of immune response					
	Th1	Th2	Cross primming	B-cell	Mucosal	Persistent T-and B-cell
Mineral Salts (aluminium salts, calcium phosphate, AS04 [Alum+MPL®]	+	++	-	+++	-	+
Emulsions [MF59^{IM}(squalene/water), QS21,AS02(squalene+MPL®+QS21), IFA, Montanide®, ISA51, Montanide®, ISA720]	++	-	-	+++	-	-
Liposomes(DMPC/Chol, AS01)	+++		+	+	-	++
Virosomes(IRIV), ISCOMs	++	++	++	+++	-	-
DC Chol, mineral oil, IFA, Montanide®, squalene	-	++	-	+++	-	-
Mucosal delivery systems:Chitosan	-	-	-	-	-	++
Microspheres	+	-	++		-	-

2.7.1 Freund`s adjuvant

For half a century, incomplete (IFA) and complete Freund´s adjuvant (CFA) have been the most commonly used immunoadjuvants for experimental work [158]. It is generally assumed that incomplete (IFA) and complete Freund´s adjuvant (CFA) act by prolonging the lifetime of injected antigens, by stimulating its effective delivery to the immune system and by providing a complex set of signals to the innate compartment of the immune system, resulting in altered leukocyte proliferation and differentiation.

Freund had suggested three categories of action mechanisms: 1) prolongation of the presence of antigens at the site of injection, 2) more effective transport of the antigens to the lymphatic system and to the lungs, where the adjuvant promotes the accumulation of cells concerned with the immune response, and 3) other mechanisms that should remain unidentified, because their clarification would require knowledge about how antibodies are formed and how sensitization develops. Briefly, later studies on some mechanisms of action for Freund´s adjuvant were categorized as follows; i. Enhancment of antigen uptake by APCs, ii. Emission of danger signals resulting in Th1 skewing, iii. Cytokine induction, iv. Chemokine induction, v. Granuloma formation, and vi. Expansion and subsequent contraction of activated $CD4^+$ T cells.

As evident from the reviewed evidence, primary target cells for the adjuvant components are mononuclear phagocytes and dendritic cells, which can produce TNF-α, IL-12, and IL-6. Early IFN-γ may come from NK cells, which may become involved as soon as IL-12 appears on the scene but may also be triggered more directly through a pathway involving their activating receptor, NKGD2, which recognize MHC-I-like antigens induced on several cells by stress signals [159]. Production of TFN-α can be presumed to play a role as inducer of other cytokines (such as IL-6) and chemokines. IL-6 may also play a role as stimulator and activator of T lymphocytes. A summary of the proposed actions of Freund´s on chemokine induction are illustrated in figure 5.

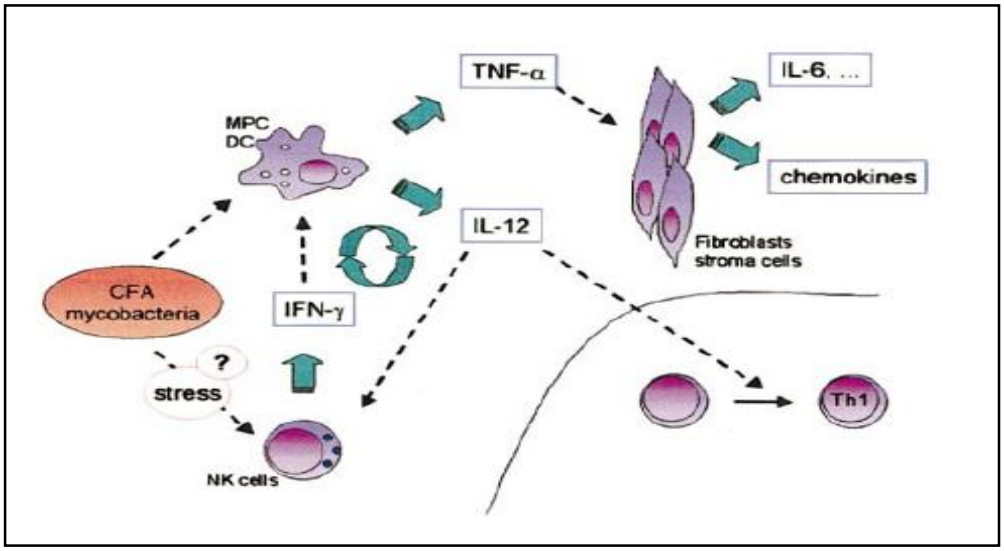

Figure 5. Cytokines observed to be induced in the early phases following exposure to CFA (or mycobacteria) are TNF-α, IL-12, IL-6, IFN-γ, and several chemokines. Mycobacterial components are known to target mononuclear phagocytes and dendritic cells (involving toll-like receptors) and to induce production of monokines, in particular IL-12 and TNF-a. IL-12 induces NK cells to produce IFN-γ, which potentiates production of IL-12, forming a positive feedback loop (curved arrows). More direct stimulation of NK cells might take place via their activating receptor NKGD2, which recognizes stress-induced membrane ligands. TNF-a can be presumed to play a role as inducer of other cytokines (such as IL-6) and of chemokines. IL-12 is the driving force for directing T-cell differentiation to assume a Th1 profile. (Adapted from [158]).

2.8 Antibody purification

2.8.1 Structure of an Immunoglobulin molecule

Antibody (or immunoglobulin) molecules are glycoproteins composed of one or more units, each containing four polypeptide chains: two identical heavy chains H and two identical light chains L (Figure 6).

The amino terminal ends of the polypeptide chains show considerable variation in amino acid composition and are referred to as the variable V regions to distinguish them from the relatively constant C regions. Each L chain consists of one variable domain VL and one constant domain CL. The H chains consist of a variable domain, VH, and three constant domains CH1, CH2 and CH3. Each heavy chain has about twice the number of amino acids and MW (~50,000) as each light chain (~25,000), resulting in a total immunoglobulin MW of approximately 150,000. Heavy and light chains are held together by a combination of noncovalent interactions and covalent interchain disulfide bonds, forming a bilaterally symmetric structure. The V regions of H and L chains comprise the antigen-binding sites of the immunoglobulin (Ig) molecules. Each Ig monomer contains two antigen-binding sites and is said to be bivalent. The hinge region is the area of the H chains between the first and second C region domains and is held together by disulfide bonds. This flexible hinge region allows the distance between the two antigen-binding sites to vary (Figure 6).

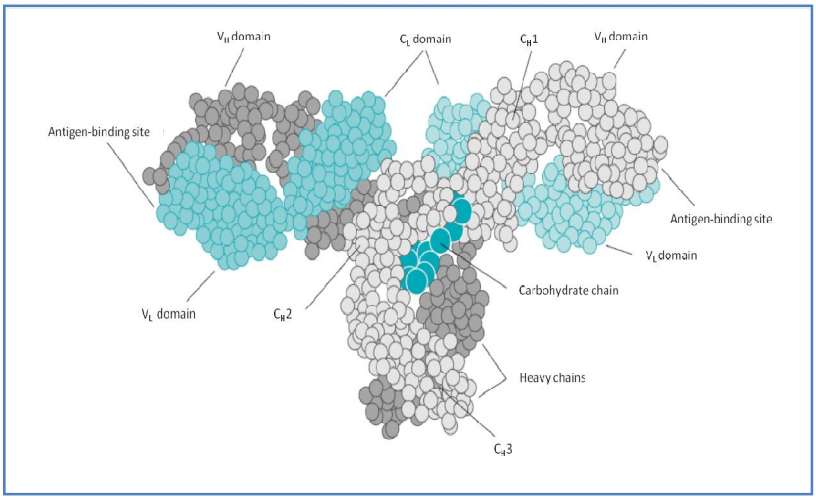

Figure 6. The three-dimensional structure of IgG can be depicted as a Y-shaped molecule. Each sphere represents an amino acid residue. The light chains are shades of blue and the two heavy chains are shades of gray. (Adapted from www.wiley.com/legacy/products/subject/life/elgert/CH04.pdf).

2.8.2 Affinity purification of IgG using protein A/G

Crude purification of antibodies can be accomplished by methods such as ammonium sulfate precipitation, thiophilic adsorbtion or affinity chromatography. In affinity chromatography (affinity purification), a ligand is coupled to a solid support material such as agarose gel. Sample fluids are passed through the support material, allowing immunoglobulins to bind to the immobilized ligand. After non-bound sample components are washed from the support, washing buffer conditions are altered so that the immunoglobulins are dissociated (eluted) from the immobilized ligand and recovered from the support in a purified form. Protein A and protein G are bacterial cell wall components that bind primarily to the Fc region of immunoglobulins and are by far the most accepted choices for affinity purification of IgG [160].

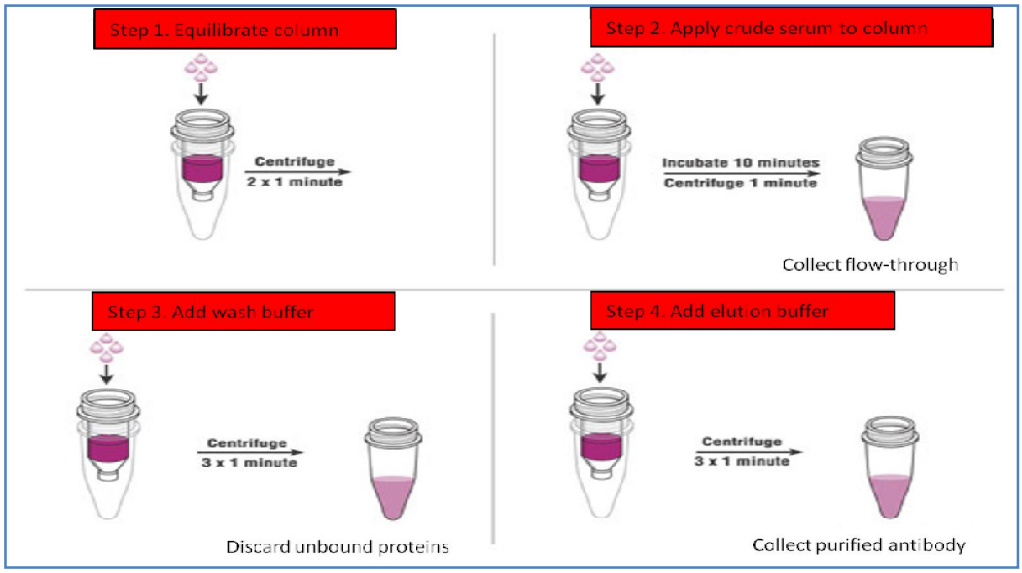

Figure 7. Purification scheme using protein A/G Nab spin kit column.

2.9 Immunoprecipitation and antigen purification

Many protocols rely on immunoprecipitation as the choice method for isolating small amounts of antigen or target protein from complex samples such as cell lysates [161, 162], serum [163, 164] and tissue homogenates [165, 166]. IP can be used to evaluate the differential expression of a protein and to characterize the proteins molecular weight, postranslational modifications, and interacting ligands. The antibodies used may be polyclonal or monoclonal, and may recognize the protein of

interest, a particular postranslational modification [167, 168], or an epitope tag [169, 170] if the protein is overexpressed. The standard method for immunoprecipitation (IP) requires the incubation of the antibody with the sample containing the target protein (antigen). Once the antigen-antibody complex is formed, it is bound to Protein A or Protein G beads (typically cross-linked agarose) via the Fc region of the IP antibody. The beads and the sample are centrifuged to pellet the captured immune complex, the supernatant discarded and the beads washed and centrifuged again to remove any unbound proteins. For high-throughput applications, immobilized Protein A or G magnetic beads can be used to facilitate separation of the isolated immune complex from the remainder of the sample. The resin pellet is then exposed to denaturing conditions or low-pH conditions to dissociate the complex. The released proteins are typically analyzed via one-dimensional [171, 172] or two-dimensional gel [173] electrophoresis followed by mass spectrometry [171, 173] or immunoblotting [174, 175]. Both the antibody and target protein are released in the final step of the traditional IP technique. This can present a problem for 1D or 2D analysis of the target protein when the molecular weight is similar to the heavy chain or light chain of the IP antibody [176].

For this reason, alternative methods have been developed to prevent the antibody from contaminating the target protein, including crosslinking the IP antibody to Protein A or Protein G beads [177, 178] or directly coupling the antibody to an activated support to create an immunoaffinity resin [179, 180], (Figure 8 and 9).

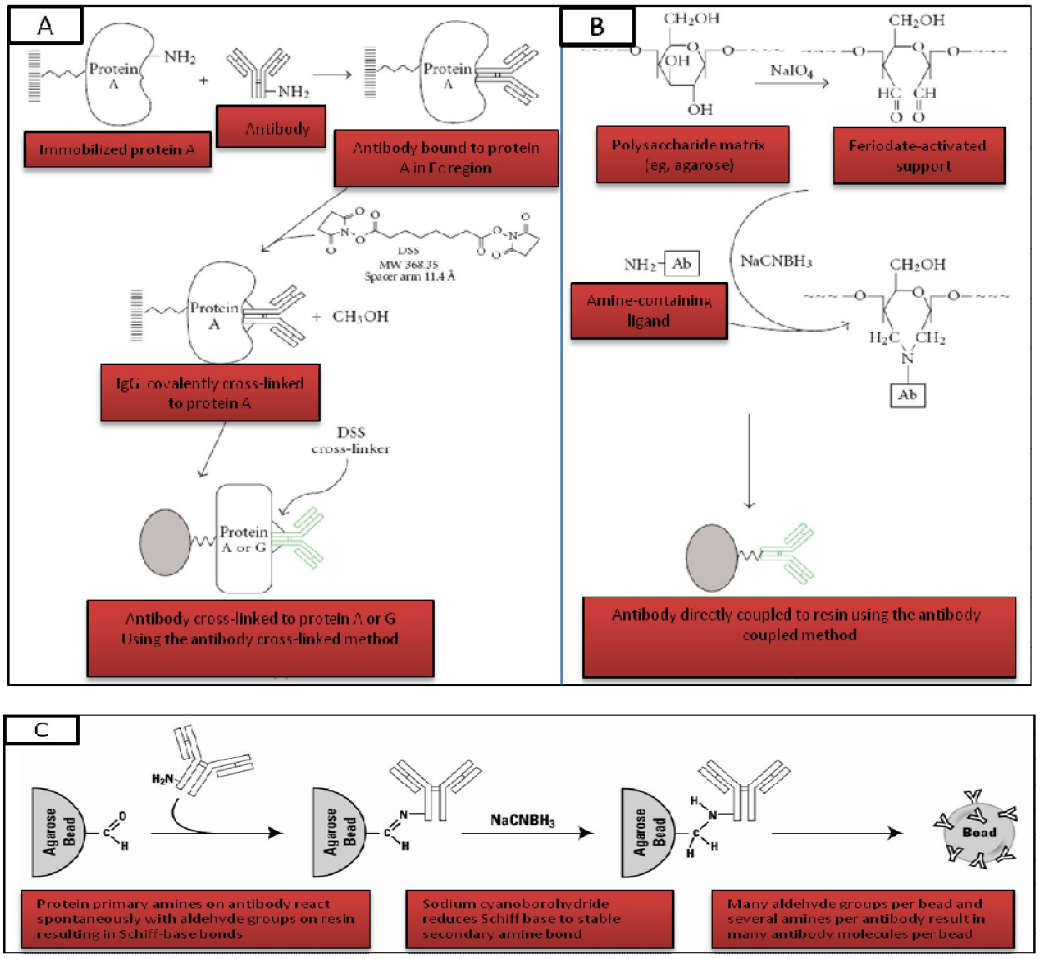

Figure 8. Diagram of the antibody bioconjugation chemistry. (A) Description of the antibody cross-linked method. (B) Schemes of the antibody-coupled method (Adopted from [176]). (C) Diagram of AminoLink reaction chemistry (reductive amination), a method for covalent, direct immobilization of IP antibody. The Pierce direct IP kit uses this method.

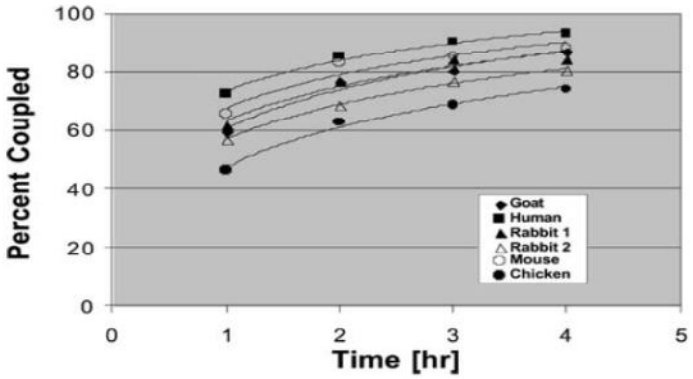

Figure 9. Antibody coupling efficiency using the direct antibody immobilization method. Purified antibody (200 µg) from various species was coupled to 200 µl of AminoLinkCoupling Gel (settled gel) at room temperature for 4 h. For the chicken antibody, 500 µg was used (Adapted from[181]).

Some of these IP strategies have also become an effective tool in studying protein: protein interactions [176, 182, 183]. Co-immunoprecipitation (Co-IP) uses the antigen–antibody complex to isolate unknown proteins bound to the antigen ("bait-prey" complex). Co-IP is a valuable *in vitro* tool to verify receptor-ligand or enzyme-substrate interactions, to identify multiprotein complex formation and to confirm yeast two-hybrid results [169, 170]. As in IP, identifying and characterizing these unknown interacting proteins can be hampered by the presence of contaminating antibody heavy and light chains (Figure 10). Therefore, the advantages of the antibody immobilization methods outlined here also readily apply to Co-IP experiments.

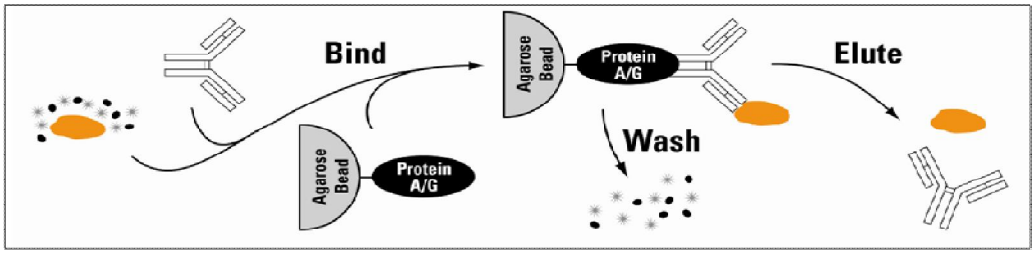

Figure 10. Diagram of a basic immunoprecipitation procedure. Antigen-containing sample (usually a cell lysate), antibody and beaded agarose affinity beads (usually Protein A or G) are allowed to bind. Non-bound sample components are washed away, and then antibody and antigen are eluted with a buffer that disrupts the binding interactions. If reducing SDS-PAGE sample buffer is used for elution, antibody and antigen products will be denatured and reduced to component polypeptide fragments.

2.9.1 Factors Affecting Immunoprecipitation

Although IP methods are logically and procedurally simple, the variables and factors affecting success of any specific experiment are as numerous and peculiar as the specific differences between different individual proteins and different primary antibodies. Immunoprecipitation involves purification of a protein or complex of proteins using specific binding conditions. Empirical testing is nearly always required before IP conditions can be optimized to successfully isolate adequate amounts and purity of specific protein. Nevertheless, consideration of the main factors involved can help to identify the components that are most likely to affect particular experiments. A list of such factors and associated variables is described in (Table 4), followed by a discussion of several pertinent issues [4].

Table 4. Factors that affect assembly of the purification complex. (Adapted from Immunoprecipitation technical guide and protocols, Thermo scientific).

Factor	Variable Characteristic
Method Format	Column vs. batch method; spin vs. gravity columns
Type of support	Physical characteristics, capacity, non-specific binding
Immobilization of Antibody	Amount, orientation, method of attachment
Immoilization of Bait	Tag, affinity ligand
Order of Addition	For beads, antibody/bait and antigen/prey
Lysate Pre-clearing	Non-specific binding
Binding Buffer	Components, stringency
Wash Buffer	Components, stringency
Elution Buffer	Components, elution strength

Immunoprecipitation as performed by the batch method simply involves mixing the components of the reaction in a reaction vessel (usually a microcentrifuge tube) for a period of time to allow them to interact. At each step, the beads are separated from the solutions (nonbound sample, wash buffer and finally elution buffer) by centrifuging the tube to pellet the beads and carefully pipetting to remove the supernatant.

Column methods involve incubating IP components with beaded resin that is packed in a plastic or glass column. The sample is either allowed to pass the column by gravity or centrifugation or the column is capped and the sample incubated with the resin (with optional mixing) to allow the antibody and antigen more time to bind. In either case, the sample solutions are separated from the beads by gravity-flow or centrifugal collection from the column tip.

Large scale IPs (>10 ml resin) are generally limited to gravity-flow because of the impracticality of centrifuging large columns, especially if they are not designed to fit in a collection tube. Conversely very small scale applications require centrifugation, as just a few microliters of solution will not flow through a filter by gravity alone. Most medium scale IPs can be performed by either gravity-flow or centrifugation so long as suitable columns and collection tubes are available, and the beaded support is compatible with the increased pressures associated with centrifugation.

The use of spin columns has a distinct advantage over both gravity columns and batch methods because almost all of the residual solution can be spun through the filter allowing cleaner separation of the solid and aqueous phases. Gravity columns

require constant monitoring to make sure the resin does not run dry and form air bubbles. In addition the antigen is eluted in multiple fractions, each of which must be monitored for the presence of antigen. Fractions containing antigen are normally pooled, therefore the volume will end up being much greater than the original sample and the antigen may require concentration. A disadvantage of the batch method is the formation of the resin pellet, which contains a significant volume of solution that cannot be removed by pipetting; additional wash and elution steps are necessary to obtain good purity and yield.

2.10 Protein oxidation as a tool to identify new antimalarial antigens for vaccine development

Upon Plasmodium infection of the RCB, both host and parasitic cells suffer significant changes in the pattern of protein oxidation, likely derived from the oxidative stress imposed by the processes associated to the metabolism of haemoglobin. Some of these changes have been recently characterized and associated with protection against malaria in Glucose-6-phosphate-deficient [184] and blood group O populations [185]. The identification of antibodies targetting carbonylated epitopes in the erythrocyte or the parasite in infected mice may thus be valuable to enrich the variety of candidate antigens suitable for vaccine formulations.

Reactive oxygen species (ROS) are constantly generated within cells at low concentrations under physiological conditions, playing a part in the cellular redox regulation. Cellular production of ROS occurs both from enzymatic and non-enzymatic sources. ROS can also occur as the outcome of acute cell stresses and may result in cell death via apoptosis or necrosis. Cellular oxidative damage developes when the balance between ROS-generating systems and ROS-scavenging ones tilts in favour of the former. The primary cellular target of oxidative stress can vary depending on the cell type, the absolute level and duration of oxidant production, the species of ROS generated, its site of generation (intra- vs. extra-cellular), and the proximity of the oxidant to a specific cellular substrate. Proteins are major targets for ROS and secondary by-products of oxidative stress when these are formed *in vivo* either in intra- or extracellular environments, as they are the major component of most

biological systems and can scavenge 50-75% of reactive radicals such as •OH [186]. There is increasing evidence that accumulation of oxidized dysfunctional molecules in the cell over a lifetime contributes to the reduction of its half-life [187, 188].

Some ROS-induced protein modifications can result in unfolding or alteration of protein structure, and some are essentially harmless events. Irreversible modifications such as protein carbonylation, and protein-protein cross-linking are generally responsible for permanent loss of function of the damaged proteins which are subsequently degraded or may progressively accumulate in intra-cytoplasmic inclusions as observed in some neurodegenerative disease [189]. In fact, such permanent modifications are implicated in the etiology or progression of a number of disorders and disease [190].

2.10.1 Protein carbonylation and carbonyl group detection

Ros-mediated protein carbonylation is an important marker of protein oxidation and its measurement is thought to be a good and the most widely used indicator for the extent of oxidative damage of proteins associated with various conditions of oxidative stress, aging and physiological disorders [191].

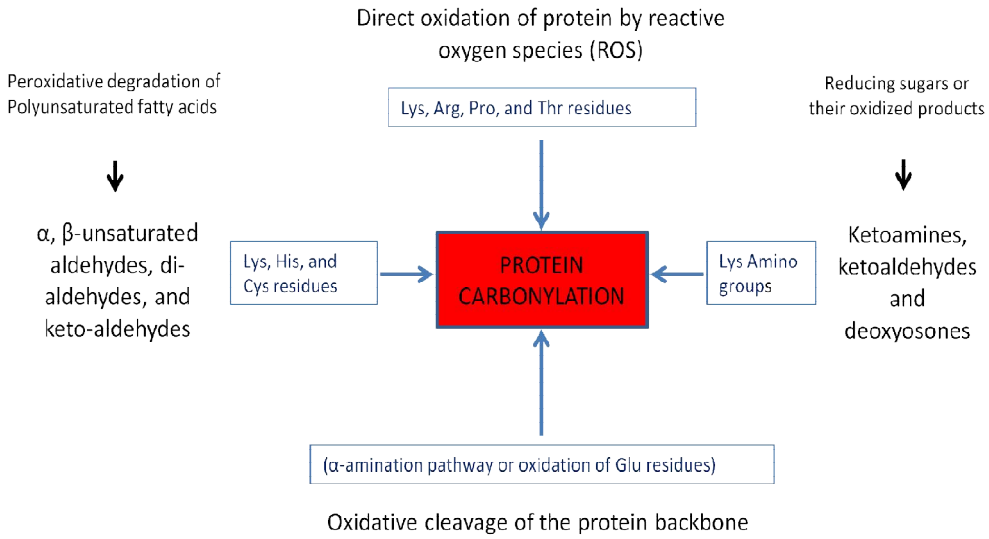

Figure 11. Origins of carbonylated proteins. Protein carbonyl derivatives can be produced by different oxidative pathways. ROS can react directly with the Lys, Arg, Pro, and Thr side chains mainly through metal-catalysed oxidation (pathway 1). Direct oxidation of proteins by ROS can also yield highly reactive carbonyl derivatives resulting from the cleavage of peptide (pathway 2). Carbonyl groups may be introduced into proteins by adduction of reactive aldehydes derived from the metal-catalysed oxidation of polyunsaturated fatty acids (pathway 3). These lipoxidation products include α,β-unsaturated aldehyes which can undergo Michael-addition reactions and react with the sulfhydryl group of Cys, the ε-amino group of Lys or the imidazole group of His residues, di-aldehydes and γ-ketoaldehyes which react with Lys residues. Finally, carbonyl groups can also be generated by secondary reaction of the primary amino group of Lys residues with reactive carbonyl derivatives (ketoamines, ketoaldehydes, deoxyosones), produced by the reaction of reducing sugars or their oxidized products with lysine residues of proteins (pathway 4) [192].

2.10.2 Determination of protein carbonyl groups

The classical approach to the detection of protein carbonylgroups involves their reaction with 2,4-dinitrophenylhydrazine (DNPH) followed by the spectrophotometric quantification of the acid hydrazone at 370 nm [193-195]. While this method has provided much valuable data on the correlation of carbonyl formation with oxidative stress, it has the disadvantage of requiring relatively large (up to milligram) amounts of protein. Furthermore, the limited solubility of many DNP-derivatized proteins, and difficulties in eliminating free DNPH from the derivatized proteins [196], makes this method prone to interference [197, 198]. Other methods for carbonyl analysis include HPLC techniques [194, 199] based on the spectrophotometric detection of DNP-carbonyl derivatives after separation of the proteins by gel-permeation or reverse-phase chromatography. HPLC analysis is useful for investigating purified proteins but is less useful in crude mixtures where problems with resolution

make it especially difficult to analyze low- and medium-molecular-weight proteins [200]. Carbonyl groups can also be detected by labeling with tritiated borohydride [193]. This technique is highly sensitive and specific when applied to samples of purified proteins, but high backgrounds and poor specificity (as tritiated brohydride also react with Schiff´s bases) can complicate its application to unfractionated tissue supernatant.

Immunochemical techniques have been also applied to the detection of carbonyl groups in proteins separated by polyacrylamide gel electrophoresis [201, 202]. Applying this approach, it is important to have reliable methods to identify specific oxidized proteins. It has been previously described an analytical procedure that allows both the reversible staining of total proteins and the specific immunostaining of the oxidized proteins separated on polyacrylamide gels [203]. However, a shortcoming of the procedure is that it requires the protein mixture (e.g., cell or tissue extract) be pre-derivatized prior to electrophoretic separation. For example, the protein derivatization of carbonyl functional groups with 2,4-dinitrophenylhydrazine (DNPH) is used for antibody recognition of oxidized proteins. Unfortunately, this pre-derivatization alters the electrophoretic (and electrofocusing) properties of proteins. Consequently, it is not possible to directly compare the patterns from oxidized fingerprints with those from non-oxidized protein fingerprints that have been compiled into large database. The ability to conduct all derivatization and staining after electrophoresis and transblotting would offer many additional advantages over pre-drivatization procedures (e.g. determining oxidation of samples that have been blotted for prior experiments). Hence in the 3rd chapter of this work, we have applied a post-electrophoretic identification of oxidized proteins that could permit comparison of oxidation proteins with those that could identified with resistant ICR mouse serum to malaria [184, 204, 205].

2.10.3 Analysis of proteins by mass spectrometry (MS)

Among the analytical techniques, MS holds a special place because it measures an intrinsic property of a molecule, its mass, with very high sensitivity and therefore it is used in an amazingly wide range of applications.

Biomolecules being large and polar, however, they are not easily transferred into the gas phase and ionized. Electrospray (ES) and matrix-assisted laser desorption ionization (MALDI) are the ionization techniques that should be credited most for the success of mass spectrometry in the life sciences. Currently, the uses of MS in proteomics are in three major areas. MS is the preferred technique for characterization and quality control of recombinant proteins and other macromolecules, an important task in the field of biotechnology. It is also commonly used for protein identification, either in classical biochemical projects or in large-scale proteomic ones. Finally, because MS measures the molecular weight of a protein, it is the method of choice for the detection and characterization of posttranslational modifications and potentially can identify any covalent modification that alters the mass of a protein.

OBJECTIVES

3 OBJECTIVES

Data in animal models support the idea of developing a malaria vaccine that can improve on natural immunity by either inducing more robust and potent immune response against a selected panel of antigens recognized as immunodominant in the context of natural infection or inducing a broader immune response against a large number of parasite antigens. Further, naturally acquired immunity in human and animals can be used as models for the development of a vaccination strategies to prevent infection or death and severe disease. However, antigens and epitopes involved in this type of protection are largely unknown since remain unclear which of them correlates with protection after experimental immunization or natural exposure . The challenge for next generation malaria vaccine is to identify which of the many antigen-specific responses might be predominantly responsible for protection, and selection of these antigens is needed for vaccine development.

Various studies in our laboratory, have shown that ICR mice populations are partially resistant to infection with lethal doses of *Plasmodium yoelii* 17XL (*PY*17XL). Remarkably, surviving ICR mice become fully resistant to PY17XL, showing an enhanced antibody response after a second challenge. These surviving mice could therefore be a suitable source of immunoglobulins for the identification of antigenic plasmodial proteins. In the other hand, upon plasmodium infection of the RBC, both host and parasitic cells suffer significant changes in the pattern of protein oxidation likely derived from the large amounts of reactive oxygen species (ROS) generated by the active metabolism of the multiplying malaria parasite and metabolism of hemoglobin. Hence, the identification of targeting carbonylated epitopes in the erythrocyte or the parasite in infected mice may help to identify new candidate antigens suitable for improved vaccine formulation.

To pursue the previous hypothesis the research work here described was designed according to the following 3 objectives:

1- To develop a procedure designed to isolate and identify plasmodial proteins displaying strong reactivity against antibodies from naturally hyper immunized mice resistant to blood-stage malaria.

2- To examine functionally the multiple affinity purified blood-stage parasite antigens from objective 1 with respect to their potential to induce protective immunity against lethal blood-stage malaria infection.

3- To identify several novel posttranslational carbonylated antigens during the blood-stage life cycle of *Plasmodium yoelii* 17XL, by using DNPH derivatization of oxidized protein and comparatively to identify several plasmodial potential vaccine antigens using malaria-resistant mice sera.

RESEARCH WORK

4 RESEARCH WORK

4.1 CHAPTER 1

Plasmodium yoelii blood-stage antigens newly identified by immunoaffinity using purified IgG antibodies from malaria-resistant mice

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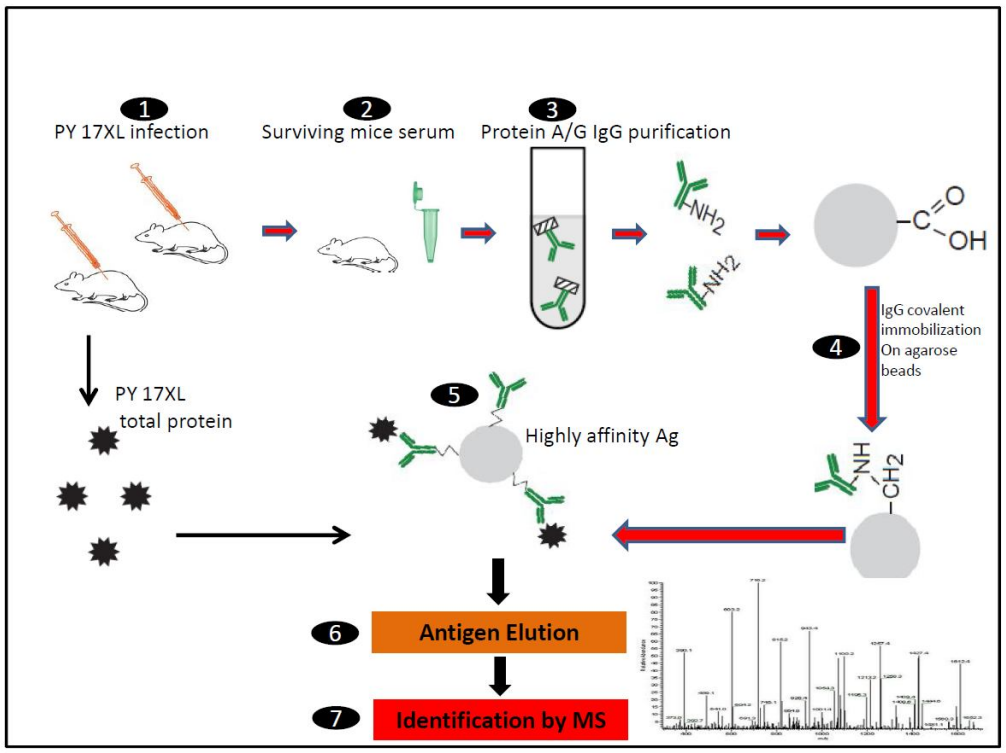
GRAPHICAL ABSTRACT

Running title; New *Plasmodium yoelii* blood-stage antigens identified



In this study, methods were designed to isolate and characterize plasmodial proteins displaying strong reactivity against antibodies from naturally immunized mice. The approach is useful for the identification of novel antigenic targets for new vaccine therapies. Interestingly, the system could be easily adapted to the identification of protective antigens recognized by the immune system in human acquired immunity to malaria or other infectious disease.

ABSTRACT

As the search for an effective human malaria vaccine continues, understanding immune responses to Plasmodium in rodent models is perhaps key to unlocking new vaccine strategies. The recruitment of parasite-specific antibodies is an important component of natural immunity against infection in blood-stage malaria. Here, we describe the use of sera from naturally surviving ICR mice after infection with lethal doses of *Plasmodium yoelii yoelii* 17XL to identify highly immunogenic blood-stage antigens. Immobilized protein A/G was used for the affinity-chromatography purification of the IgGs present in pooled sera from surviving mice. These protective IgGs, covalently immobilized on agarose columns, were then used to isolate reactive antigens from whole *P. yoelii yoelii 17XL* protein extracts obtained from the blood-stage malaria infection. Through proteomics analysis of the recovered parasite antigens, we were able to identify two endoplasmic reticulum lumen proteins: protein disulfide isomerase and a member of the heat shock protein 70 family. Also identified were the digestive protease plasmepsin and the 39 kDa-subunit of eukaryotic translation initiation factor 3, a ribosome associated protein. Of these four proteins, three have not been previously identified as antigenic during blood-stage malaria infection. This procedure of isolating and identifying parasite antigens using serum IgGs from malaria-protected individuals could be a novel strategy for the development of multi-antigen-based vaccine therapies.

Keywords

Malaria; Immunoglobulins; Mass spectrometry; Antigens; Protein disulfide isomerase; Translation initiation factor 3; Plasmepsin; Heat shock protein.

INTRODUCTION

Detailed understanding of the immune response in individuals who develop naturally acquired immunity to malaria infection could help identify valuable antigen candidates for new vaccine formulations. It is well established that B cells play a crucial role in the naturally acquired immunity to malaria observed in individuals living in endemic areas. This kind of immunity, which takes years to develop, is largely dependent on the acquisition of specific, protective antibodies directed against a repertoire of target antigens [1, 2]. Over the past years, the use of naturally resistant, susceptible, and genetically deficient mice, together with different Plasmodium strains has enabled the dissection of some of the immunological mechanisms developed against *Plasmodium* spp. infection in rodent models [3-6]. From these and many other studies, it is clear that both cellular and humoral immune responses are required to control and clear blood parasitemia. Nevertheless, it is well known that infections vary in virulence depending on the species and strain of both the infectious Plasmodium agent and the host rodent. Thus, different strains of inbred mice elicit antimalarial antibodies of diverse antigenic specificities [7]. Moreover, after primary malaria infection, serum samples collected on different days show distinct patterns of immunoreactivity [8], indicating temporal immunological modulation during infection.

In non-lethal infections (e.g., those caused by *P. chabaudi chabaudi* or *P. yoelii* 17 XLNL), recovery from infection generally results in immunity to a second challenge by the same strain or species of the parasite but not by a heterologous parasite [4]. Interestingly, the highly virulent L (lethal) strain of *Plasmodium yoelii yoelii* 17X (PY17XL) appears to activate Th2 but not Th1 cells, while the low-virulence NL (non-lethal) strain activates both subsets of $CD4^+$ T cells [9]. During *P. yoelii* infection, Th1 cells producing interleukin-2 (IL-2), interferon-gamma (IFϒ) and tumor necrosis factor-ß (TNF-ß) activate macrophages to kill pathogens whereas Th2 cells, producing IL-4, IL-5 and IL-10, assist B cells in producing antibodies [10]. Previous results from our laboratory have shown that ICR mice populations are partially resistant to infection with lethal doses of PY17XL [11]. Remarkably, surviving ICR mice become fully resistant to PY17XL, showing an enhanced antibody response after a second challenge [11].

These surviving mice could therefore be a suitable source of immunoglobulins for the identification of antigenic plasmodial proteins.

Immunoaffinity chromatography uses immobilized antibodies as affinity ligands specific to either an antigen or a group of structurally-related antigens [12]. In this study, we developed a procedure designed to isolate and characterize plasmodial proteins displaying strong reactivity against antibodies from naturally immunized mice. This approach is useful for the identification of novel antigenic targets for new vaccine therapies. Moreover, the system could be easily adapted to the identification of protective antigens recognized by the immune system in human acquired immunity to malaria or other infectious diseases.

MATERIALS AND METHODS

Malaria parasites and animals.

The rodent malaria parasite *Plasmodium yoelii yoelii* 17XL (PY17XL) was kindly provided by Dr. Virgilio Do Rosario (Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa) and stored after serial blood passages in mice. Infected blood was kept in liquid nitrogen in a solution containing glycerol 28% (v/v), sorbitol 3% (w/v) and NaCl 0.65% (w/v). Random-bred ICR female mice (Hsd:ICR[CD-1]), aged 6-8 weeks, were purchased from Harlan Laboratories. The mice were housed under standard conditions of light and temperature in the animal house of the Universidad Complutense de Madrid (Madrid, Spain). *In vivo* experiments were approved by the Animal Experimentation Committee of this institution.

For parasite isolation and immune serum sampling, mice were inoculated by intraperitoneal injection of 10^7 red blood cells from *P. yoelii* -infected mice in 0.1 mL phosphate buffered saline (PBS). After infection, *p*-aminobenzoic acid at a final concentration of 0.05% (w/v) was added to the drinking water [13]. Parasitemia was monitored daily by microscopy examination of Wright's-stained thin blood smears. To assess protective immunity, the cured mouse fraction (about 20%) that spontaneously cleared the blood parasites was challenged with the same dose of parasites 40 days after their full recovery from the primary infection, and parasitemia was further monitored for 30 days as previously described [11]. Mice surviving this second challenge were considered malaria-resistant mice.

Purification of mouse IgGs.

IgGs from 150 µL-pooled serum samples of malaria-resistant mice were specifically bound onto a 0.2 ml NAb™ protein A/G column (Pierce) according to the manufacturer's instructions. Briefly, binding buffer (sodium phosphate 100 mM containing NaCl 150 mM, pH 7.2) was used as diluent and as the binding and wash buffer. Bound IgGs were eluted in 400 µL fractions using the IgG elution buffer, pH 2.8 provided. Collecting tubes were previously preloaded with 40 µL of Tris-HCl 1 M, pH 8.5 for neutralization. Since amines (e.g., Tris) in the IgG fraction compete for coupling

sites on the resin for subsequent immobilization (see below), they should be removed by dialysis. Thus, each 1.2 mL fraction of purified mouse IgGs was dialyzed in a Slid-A-Lyzer™ dialysis cassette (Thermo Scientific) against 500 mL of sodium phosphate 0.01 M containing NaCl 0.15 M, pH 7.2 for 2 h, with a total of 3 replacements performed. After the third replacement, equilibrium was continued overnight at 4°C. Aliquots of 50 µg (100 µL) of purified and dialyzed mouse IgGs were kept at -20°C until their use.

Immobilization of mouse IgG.

Immunoaffinity columns were prepared by immobilization of IgG antibodies from resistant ICR mice on agarose-loaded spin columns (Pierce Direct IP kit cat. n. 26148)[14]. Briefly, 100 µL of AminoLink Plus coupling resin were applied to each column and centrifuged at 1,000 ×g for 1 min. The columns were then washed twice with 200 µL of 1x coupling buffer (sodium phosphate 0.01 M containing NaCl 0.15 M pH 7.2). Next, 50 µg of purified IgGs from malaria-resistant mice were loaded onto the column and the volume immediately adjusted to 200 µl using ultrapure water and 20x coupling buffer to give a final concentration of 1x coupling buffer. After addition of 3 µL of sodium cyanoborohydride 5 M to allow covalent binding, the column was capped and incubated at room temperature with rotation for 2 h. Next, the column was washed twice with coupling buffer and pre-washed with 200 µl of quenching buffer (Tris-HCl 1M) to remove any uncoupled IgG. To block the remaining sites on the resin, the column was bottom capped again, 200 µL of quenching buffer plus 3 µL of sodium cyanoborohydride were once again added, and the column incubated for 15 min with gentle shaking. Finally, the column was washed twice with coupling buffer, 6 times with wash solution (NaCl 1M) and subjected to a final wash with TBS (Tris-buffered saline, Tris 0.025 M, NaCl 0.15 M; pH 7.2).

Parasite extracts.

P. yoelii-infected ICR mice were sacrificed under anesthesia when the parasitemia level averaged 50%. Blood containing a mixture of ring-, trophozoite- and schizont-stage parasites was collected in 0.1 M EDTA and kept at -80°C until use. For

total protein extraction, 5 mL of infected RBC were lysed with 0.1% saponin. The intact parasites were collected by centrifugation at 320 ×g for 5 min and washed with cold PBS by centrifugation at 7,800 ×g for 15 min until the supernatant was colorless. Parasite pellets were resuspended in extraction buffer (Tris-HCl 50 mM pH 8.0, NaCl 50 mM, CHAPS 3%, Mega-10 0.5% and 1 tablet of complete Mini Protease Inhibitor Cocktail from Roche per 10 mL of buffer) and homogenized using a mini-potter for 10 min at 4°C followed by 4 cycles of freezing (-20°C) and thawing (37°C). Homogenates were centrifuged at 20,000 ×g for 15 min and the supernatant recovered as the parasite whole protein extract and kept at -80°C until use.

Isolation of parasite antigens by immunoaffinity.

Total proteins extracted from PY17XL (500-1000 µg) in a 600 µL volume of TBS were loaded onto an antibody-coupled spin column and incubated for 2 h with gentle shaking. To remove non-bound proteins, the complex was washed three times with TBS and once with conditioning buffer (neutral pH, supplied with the Pierce Direct IP kit, Cat. No. 26148). Sodium deoxycholate 1% (w/v) (Sigma-Aldrich) in PBS was used to dissociate the bound antigens from the immobilized antibody and the eluate was recovered in PBS [15].

Protein determination.

The Bio-Rad protein assay (Cat. No. 500-0006) and Bio-Rad DC protein assay (Cat. No. 500-0116) were used to determine protein concentrations in whole parasite extracts and in the solutions of purified antigens, respectively.

Immunoblotting.

Parasite total protein extracts, prepared as described above, were solubilized in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing SDS 2.5 %, boiled for 5 minutes and subsequently separated on 10% SDS-PAGE. After electrophoresis, proteins were transferred onto PVDF (Hybond-P, GE Healthcare)

membranes following standard procedures. Blots were blocked for 1 h with PBS containing 5% non-fat dried milk. The blots were subsequently incubated overnight with either serum or isolated IgGs from malaria-resistant ICR mice diluted in PBS containing Tween-20 0.05%. Bound IgGs were detected using HRP-conjugated anti-mouse IgG (GE Healthcare) at 1/5000 dilution. Detection was performed using the SuperSignal chemiluminescence substrate (Pierce) and exposure to X-ray film.

SDS gel electrophoresis of parasite antigens.

Parasite proteins isolated by immunoaffinity (20 µg) were diluted in 5x Tris-glycine SDS sample buffer (Tris-HCl 50 mM, pH 6.8 containing SDS 2%, bromophenol blue 0.1%, β-mercaptoethanol 5% and glycerol 10%) to obtain a final concentration of 1x Tris-glycine SDS sample buffer for optimal band resolution. Samples were loaded onto 10% gradient polyacrylamide Tris-glycine gels and size fractionated by SDS-PAGE (120 V, 90 min) followed by colloidal staining with Coomassie Brilliant Blue G-250 [16].

Tryptic digestion and MALDI-MS.

Bands of the eluted antigens separated by SDS PAGE were manually excised from the SDS-PAGE gels, deposited in 96-well plates and automatically processed in a Proteineer DP (Bruker Daltonics, Bremen, Germany) for protein reduction, alkylation, and digestion with trypsin as described elsewhere [17]. After digestion, the supernatant was collected and 1 µL was spotted onto a MALDI target plate and allowed to air-dry at room temperature. Next, 0.4 µL of α-cyano-4-hydroxytranscinnamic acid matrix (Sigma) 3 mg/ml in 50% acetonitrile were added to the dried peptide digest gel strips and allowed to air-dry at room temperature again. MALDI-TOF analyses were performed in an ABI 4700 MALDI TOF/TOF mass spectrometer (Applied Biosystems, Framingham, MA, USA) operated in positive reflector mode, with an accelerating voltage of 20 kV. Mass spectra were then collected for peptide mass fingerprinting (PMF). MS/MS data were submitting to the Mascot program version 2.2.04 (Matrix Science, London, UK) using GPS Explorer

version 4.9 to search in the nonredundant NCBI protein database (NCBInr 20090406; 8,198,267 sequences; 2,824,199,726 residues).

RESULTS

Integrity and variety of purified antibodies from malaria resistant mice.

Protein A/G is a recombinant fusion protein that includes the IgG-binding domains of both protein A and protein G, allowing the binding of a broad range of IgG subclasses from a variety of mammalian sources. To evaluate the performance of the protein A/G system for the purification and isolation of IgGs from mouse serum, pooled sera obtained from malaria resistant ICR mice were loaded onto protein A/G columns and IgGs specifically eluted. The resultant eluate was analyzed by SDS-PAGE to determine the degree of purity and homogeneity of the recovered IgG fractions. As shown in Fig. 1A, most (>85%) of the recovered proteins from the acidic elution of the A/G columns were immunoglobulins, as revealed by the MW of the two major bands corresponding to the heavy (~50 kDa) and light (~25 kDa) chains of IgG.

The integrity of the IgGs eluted from the protein A/G columns was verified by Western blotting of the purified IgG fraction and comparing this with that of the original loaded serum, further identified using anti-mouse IgG/HRP-linked F(ab). Fig. 1B reveals no difference between the purified IgG and intact serum, indicating no size changes incurred by both the heavy and light chains of the purified IgGs during the purification process with no signs observed of proteolytic degradation.

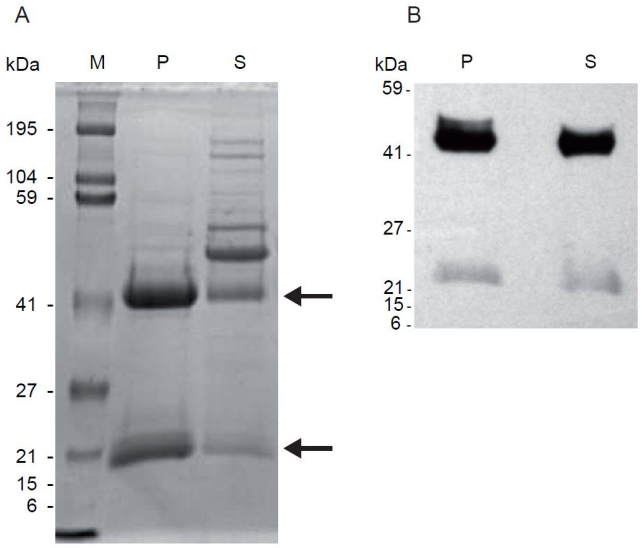


FIGURE 1. Affinity chromatography using serum protein A/G from malaria protected mice. (**A**): SDS-PAGE of serum proteins recovered after affinity purification on Nab A/G spin columns (lane P) compared to SDS-PAGE on intact serum (lane S). 10 µg were loaded per lane. M, molecular weight markers. (**B**): Western-blot analysis of purified IgGs (lane 1) and total serum (lane 2) from malaria-protected mice. 10 µg of protein were separated through SDS-PAGE, transferred to PVDF membranes and detected using the secondary antibody anti-mouse IgG/HRP-linked F(ab). Arrows indicate the positions of the heavy and light IgG chains.

We then checked the functionality and variety of the purified IgGs in terms of their specific binding to a large diversity of *P. yoelii* antigens by Western blotting of the parasite total protein. Thus, when we compared the signals produced by the purified IgGs and the crude serum IgGs (Fig 2A), the purified fraction was able to bind an identical protein pattern and range to the crude serum. This confirms that the purified IgGs retain their antigen-binding functionality and variety.

Purification and identification of parasite antigens potentially conferring malaria-resistance.

Having confirmed the wide range of antigen recognition and binding of the IgGs isolated from the malaria-resistance mice, we then covalently immobilized these purified IgGs in agarose resin. The resultant sample of IgG-agarose was evaluated for IgG integrity and functionality by Western-blotting as above, revealing similar behavior to the soluble IgGs (data not shown).

To isolate the parasite's antigens, total protein extracts of *P. yoelii* were loaded onto the covalently-bound antibody column and eluted under stringent conditions to recover high-affinity bound antigens. The eluate was first tested for the presence of IgGs possibly released from the column, due to the stringent condition used, by Western-blotting with anti-mouse IgG/HRP-linked F(ab). Since no signal was obtained (data not shown), we concluded that the IgGs were not co-eluted in the flow-through. To examine the variety of antigens isolated, the same Western blot membranes were gently washed with PBS and re-incubated with immune sera from malaria resistant mice (as primary antibody) followed by incubation with anti-mouse IgG/HRP-linked Fab (as secondary antibody). As shown in Fig. 2B, a wide range of proteins reacting with the immune sera from mice were detected, indicating that the method was able to isolate antigens by high-affinity functional binding and recognition.

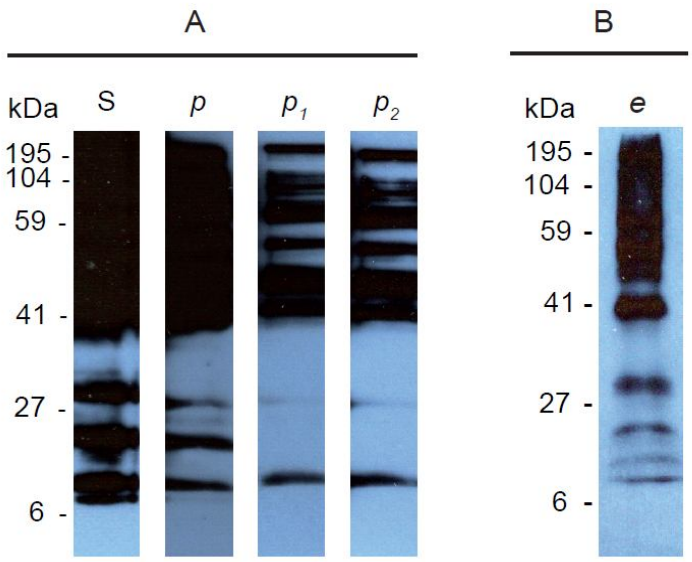


FIGURE 2. Immunoreactivity against parasite proteins. (**A**): Western blot of whole *P. yoelii* 17XL protein extracts obtained from blood-stage infection in mice. Membranes were incubated with whole serum from infected mice (lane S) or with purified IgGs (lanes *p*, *p1* and *p2*). Bands were detected using anti-mouse IgG/HRP-linked F(ab). Exposure times were 10 min (*s* and *p*), 2 min (*p2*) and 1 min (*p1*). (**B**): Western blot analysis of the eluate (*e*) obtained by immunoaffinity of total parasite proteins through immunoaffinity with immobilized IgGs from malaria-resistant mice. The membrane was first incubated with pooled immune sera from mice and then with the secondary antibody anti-mouse IgG/HRP-linked Fab. All lanes were loaded with 10 µg of protein. SuperSignal (Pierce) was used as the chemiluminescent substrate for visualization.

To identify the major protein bands in the Western blots (Fig. 2B), these were excised from corresponding duplicate gels and subjected to MALDI-TOF to yield peptide mass fingerprints. It should be noted that the concentration of sodium deoxycholate used to dissociate the bound antigens from the immobilized IgGs is not compatible with the initial isoelectrofocusing partition of 2D gel electrophoresis, and thus 1D-denaturing gel electrophoresis of the eluate fraction was conducted for proteomic analysis. Moreover, due to the limited amount of eluate recovered from the immunoaffinity flow-through columns, 2D electrophoresis could not be performed after dialysis given the large amounts of isolated IgGs and parasite proteins that would eventually be required. From the sixteen 14 to 190 kDa bands excised and analyzed by MS (Figure 3), nine proteins were identified (Table 1).

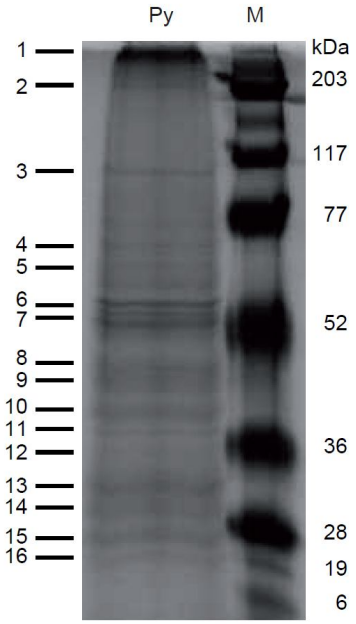


FIGURE 3. pY17XL protein bands selected for MS identification. Representative Coomassie blue stained SDS-PAGE of isolated antigens. Py: P17XL immunoaffinity purified proteins; M: molecular weight markers. Arrows indicate the bands excised for MS identification.

TABLE 1. Immunoaffinity purified antigens identified by MS/MS.

Bands	Accession n.	Mass (Da)	Score	Protein
1, 2	-	-	-	Unidentified
3	PLMN_MOUSE	93460	114	Plasminogen OS=Mus musculus GN=Plg PE=1 SV=2
4	gi|82594363	79303	98	Heat shock protein [*Plasmodium yoelii yoelii* str. 17XNL]
5	APOH_MOUSE	39904	179	Beta-2-glycoprotein 1 OS=Mus musculus GN=Apoh PE=1 SV=1
6	FIBB_MOUSE	55402	216	Fibrinogen beta chain OS=Mus musculus GN=Fgb PE=2 SV=1
7	FIBG_MOUSE	50044	172	Fibrinogen gamma chain OS=Mus musculus GN=Fgg PE=2 SV=1
8	gi|82595601	56572	81	Protein disulfide isomerase [*Plasmodium yoelii yoelii* str. 17XNL]
9	-	-	-	Unidentified
10	gi|82753379	52687	126	Plasmepsin [*Plasmodium yoelii yoelii* str. 17XNL]
11	gi|82594751	37615	67	Eukaryotic translation initiation factor 3 - 39 kDa subunit [*Plasmodium yoelii yoelii* str. 17XNL]
12	gi|82594751	37615	76	Eukaryotic translation initiation factor 3 - 39 kDa subunit [*Plasmodium yoelii yoelii* str. 17XNL]
13-16	-	-	-	Unidentified

Four of these were mouse proteins and five were of plasmodial origin. Band numbers 11 and 12 were the same *P. yoelii* protein and, thus, four different Plasmodium antigens were unequivocally identified: a member of the heat shock protein 70 family, protein disulfide isomerase, plasmepsin, and eukaryotic translation initiation factor 3. Table 2 summarizes the data obtained for these antigenic Plasmodium proteins. The spectra of the identified proteins are included as Supplementary data. MOWSE score distributions were searched against proteins by the MASCOT software and matching peptide sequences mapped over the entire identified protein sequence. For all proteins, the MOWSE score distribution was significant greater than 73 ($P<0.05$).

TABLE 2. Functional classification and protein description of identified Plasmodium proteins.

Protein Description	Database accession (Uniprot)	Annotation
Heat shock protein 70 family	Q7RER2	Molecular function: ATP binding, inferred from electronic annotation. Biological process: Response to stress. Also involved in chaperoning proteins into and through various compartments of the eukaryotic cell.
Protein disulfide isomerase	Q7RRT0	Molecular function: protein disulfide oxidoreductase activity. Biological process: cell redox homeostasis. Similarity: belongs to the thioredoxin family PFAM entry PF00085. Subcellular location: endoplasmic reticulum
Plasmepsin	Q7R9G3	Molecular function: aspartyl protease. Biological process: proteolysis. This family of aspartate proteases is classified by MEROPS as the peptidase family A1 (pepsin A, clan AA).
Eukaryotic translation initiation factor 3 39kDa subunit	Q7RRX2	Molecular function: translation initiation factor, Biological process: protein synthesis. Comments: a WD40 repeat (also known as the WD or beta-transducing repeat, PFAM entry PF00400) implicated in a variety of functions ranging from signal transduction and transcription regulation to cell cycle control, autophagy and apoptosis found in this protein.

DISCUSSION

Most researchers agree that a global understanding of the malaria parasite's biology and its associated molecular interactions with the host will help design effective antimalarial drugs and vaccines. The restricted immune response elicited by subunit vaccines featuring a small repertoire of epitopes has directed the attention of research efforts towards whole organism immunization [18-21] and multiple epitopes formulations [22, 23] to try to overcome limited immune recognition or polymorphisms in the target subunits. The search for candidate antigens based on molecular identification of essential parasite functions has also failed so far to provide effective immune protection [24].

An alternative way to achieve protection against the parasite could be the use of parasite-targeted antibodies, found in individuals who have acquired protective immunity after malaria infection, to search out new target antigens [23, 25]. To explore this approach, methodological pathways for the isolation and characterization of these antigens need to be developed. As parasite-targeted antibodies, functional Plasmodium-specific IgGs isolated from immune sera could be effective. However, the quality, quantity and functional activity of IgGs recovered from animals experimentally immunized with recombinant parasite antigens or from humans endemically exposed to malaria are highly dependent on the purification method and the species [26]. In the present work, the high-affinity chromatography system used to purify IgGs proved to be adequate for the isolation of high quality functional IgGs from malaria-resistant mice, as indicated by their electrophoretic profile and their reactivity against *P. yoelii* proteins.

IgG specificity can be exploited to search target antigens out of a complex sample mixture. Thus, the recovered IgGs were subsequently used for the purification and characterization of parasite antigens by immunoaffinity. The traditional method of immunoprecipitation by incubating an antibody with a protein sample and subsequently binding it to a protein A or G agarose matrix serves to efficiently recover target antigens [27-29]. However, immunoprecipitation followed by weak binding to agarose through protein A or G has certain intrinsic shortcomings such as the loss of insoluble antigen-antibody complexes [30], or the associated release of IgGs when

eluting the antigens [31]. In addition, if the antigens and IgG subunits are of similar molecular weight, it is difficult to separate out the immunoprecipitate [27]. The direct immunoaffinity antigen-enrichment method (Fig. 4) used here has several advantages: i) Co-elution of antigen and antibody is prevented, as the IgGs are first covalently bound to an immobilized matrix. This will also avoid the appearance of contaminating high- and low molecular weight antibody chains, which could hamper the identification of immunogenic peptides. ii) The immunomatrix can be reused several times, which is useful when working with limited immune serum. iii) The stringent conditions of elution means that antigens showing high affinity for the immobilized IgGs will be preferentially selected, increasing the chances of identifying valuable immunogenic peptides. iv) The recovered proteins are suitable for MS identification, offering the possibility of screening for new vaccine candidates.

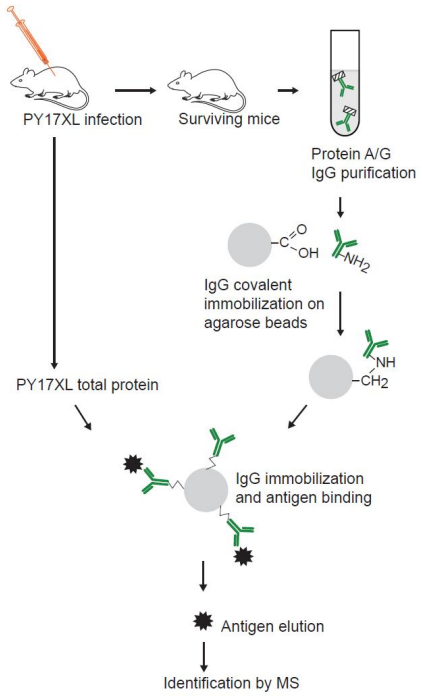


FIGURE 4. Summary of the antigen identification procedure. Sera from surviving ICR mice after Py17XL infection are used for IgG purification by protein A/G. IgG are subsequently immobilized by a procedure whereby reductive amination directly links the antibody to agarose beads. The coupling resin is provided in an activated state containing aldehyde groups formed by mild oxidation of adjacent diols using sodium meta-periodate. Primary and secondary amine groups on the antibody react with the aldehydes to form Schiff bases, which are then reduced by sodium cyanoborohydride to form secondary and tertiary amine linkages. The immobilized antibodies are thence used for purification and MS identification of antigenic peptides present in P17XL.

Using this procedure we were able to identify four highly-antigenic *P. yoelii* proteins. Remarkably, two of these proteins mainly occur in the endoplasmic reticulum (ER) lumen in the parasite: protein disulfide isomerase (PDI) and the HSPA5 member of the heat shock protein-70 family, which is also known as 78 kDa glucose-regulated protein (GRP-78) or binding immunoglobulin protein (BiP) in the corresponding mammalian homolog family. Coincidently, these two proteins are known to co-localize in the endoplasmic reticulum of rings, trophozoites, schizonts and merozoites during the intraerythrocytic growth of malaria parasites [12]. The ability of Plasmodium parasites to export proteins plays an important role in the parasite's biology, including its virulence and probably the immune response produced in the host. In the middle of this export system occurs the specific trafficking of proteins from the ER to the parasitophorus vacuole and then to the host red cell cytoplasm (reviewed in [32]). Thus, the natural exposure of parasite PDI and BiP to the host immune system, as indicated by the reacting IgGs produced in our malaria-resistant mice, suggest a role for these two proteins in the ATP-powered machinery of the Plasmodium translocon of protein export [33]. This hypothetical role is supported by the existence of a multifunctional ATP binding domain in homologous BiP [34] and the partial co-localization of PDI at the periphery of malaria parasites during red cell invasion [35]. Moreover, the affinity shown by the IgG antibodies raised in *P. yoelii*-resistant mice for PDI and BiP, identifies these two antigens as potential targets to elicit the necessary host immune responses to eliminate and control the blood-stage of the Plasmodium life cycle. It is also true that several Plasmodium antigens with a known role in the virulence and pathogenesis of malaria feature a large numbers of cysteine-residues at conserved positions within well-defined protein domains, and their correct disulfide linkage is essential for biological activity via PDI [36]. Examples of such protein domains include the F2-domain of EBA-175 [37, 38], the EGF-like domains of merozoite surface protein-1 [39], the ectodomain of apical merozoite antigen-1 [40] and host-cell-receptor binding domains on *P. falciparum* erythrocytic membrane protein 1 [41].

Although it is known that several other members of the HSP70 protein family of *P. falciparum* are immunogenic [42-46], it remains unclear whether the increased

levels of antibodies raised against these proteins are solely linked to parasite exposure [42, 46] or also to the acquisition of clinical protection [43, 45, 47].

The immunoaffinity method used here also served to identify the unique *P. yoelii* plasmepsin, a key plasmodial aspartic proteinase. In effect, this is the first time that plasmepsin has been identified as immunogenic in blood-stage malaria. The family of plasmepsins in *P. falciparum* is well defined and consists of ten members identified from the parasite's genome sequence [48]. The plasmepsin family in *P. yoelii* is smaller than in other *Plasmodium* spp., as no orthologs of PfPM1, PfPM2 and PfHAP have been detected in the genomes of any of the six other species examined, suggesting that those species, including *Plasmodium yoelii,* contain a single food vacuole plasmepsin [49]. Plasmepsins participate in the first steps of hemoglobin degradation in the digestive vacuole of malaria parasites, which is a crucial process for parasite growth. Despite their functional redundancy in *P. falciparum* [50], plasmepsins have been well characterized as potential drug targets. Indeed, functional and biochemical studies have been targeted at their inhibition aimed at halting hemoglobin digestion and eventually causing parasite death [51]. The idea that antibodies elicited against plasmepsin in our malaria resistant mice may have provided protective immunity against lethality is an attractive hypothesis. Interestingly, disruption of the single gene encoding plasmepsin-4 in the rodent malaria parasite *Plasmodium berghei* [52] generates virulence-attenuated parasites stimulating strong protective immunity against subsequent challenge with wild-type parasites.

The eukaryotic translation initiation factor 3 (IF3) was also here identified for the first time as a presenting antigen during blood-stage malaria infection. IF3 is an essential factor for eukaryotic translation and has been suggested to play a role in the fine-tuning of amino acid availability responses and under conditions where aminoacyl tRNAs are in limited supply. Modified amino acid bioavailability during Plasmodium infection causes dysregulation of L-arginine metabolism [53] and modulates NO inflammatory responses [54]. Moreover, accurate control of IF3 expression limits the premature or inappropriate abandoning of translation that would otherwise compromise cell fitness (reviewed in [55]). Thus, blocking the functionality of this

essential factor by the host immune response remains to be explored to address the immunoprotective role of this newly identified antigen.

The known antigen targets of protective host immune responses that could not be identified in our 1D electropherograms also deserve attention. Among these, merozoite surface protein 1 (MPS1) is a well-known antigen raised during protective immune responses and a potential vaccine candidate against both *P. falciparum* and other Plasmodium spp. [56, 57] MSP1 is synthesized as a precursor during schizogony and is thereafter processed on the merozoite surface into a membrane-bound, ≈19-kDa fragment, which is carried into the newly invaded erythrocyte, and a soluble ≈33-kDa fragment, which is shed from the parasite surface [58]. The protection of mice with the corresponding 19kDa fragment of *P. yoelii* MSP1 against malaria infection has been reported in this model [56]. Thus, although we could not identify MSP1 by MS in our immunoaffinity eluate, the presence of a classic MSP1 19KDa and related precursor band pattern was observed in the eluate's 1D-electrophoregram (Figure 3: bands 13 to 16). This band pattern was also clearly observed in the Western blots prepared using resistant mice serum to identify the eluted antigens (Fig.2B). Moreover, the presence of IgG-reacting MSP1 in the total proteins extracted from *P. yoelii* was confirmed by 2D immunoproteomics using ICR resistance mice serum in complementary experiments in our laboratory (data not shown).

The limited success of generating protective immunity against malaria using subunit vaccines composed of one or a few parasite proteins (or protein fragments or domains) has prompted the search for new antigenic proteins able to induce protective immunity. The circulating IgGs against blood stage malaria that maintain malaria as a subclinical disease in a proportion of the adult population exposed to the parasite constitute a major component of the immune response [1, 2]. This was the rationale behind our idea of the use of malaria-resistant mice after primary infection as a source of IgGs to search out *P. yoelii* antigens presenting during blood stage infection. Although there is still much to be learnt about protective immune responses in malaria, our results provide new direction for studies designed to identify new antigens in human malaria parasites as vaccine targets and reveal that a variety of proteins may offer efficient B cell-based protective immunity. It is tempting to suggest

that a vaccine based on such a variety of antigenic proteins elicited during the parasite blood stage could be the key to effective protection against malaria [59, 60].

ACKNOWLEDGMENTS

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REFERENCES

1. Langhorne, J., et al., *Immunity to malaria: more questions than answers.* Nat Immunol, 2008. **9**(7): p. 725-32.
2. Struik, S.S. and E.M. Riley, *Does malaria suffer from lack of memory?* Immunol Rev, 2004. **201**: p. 268-90.
3. Langhorne, J., S.J. Quin, and L.A. Sanni, *Mouse models of blood-stage malaria infections: immune responses and cytokines involved in protection and pathology.* Chem Immunol, 2002. **80**: p. 204-28.
4. Li, C., E. Seixas, and J. Langhorne, *Rodent malarias: the mouse as a model for understanding immune responses and pathology induced by the erythrocytic stages of the parasite.* Med Microbiol Immunol, 2001. **189**(3): p. 115-26.
5. Schofield, L., et al., *Gamma interferon, CD8+ T cells and antibodies required for immunity to malaria sporozoites.* Nature, 1987. **330**(6149): p. 664-6.
6. Taylor-Robinson, A.W., *Regulation of immunity to malaria: valuable lessons learned from murine models.* Parasitol Today, 1995. **11**(9): p. 334-42.
7. Taylor, D.W., et al., *Inbred mice infected with Plasmodium yoelii differ in their antimalarial immunoglobulin isotype response.* Parasite Immunol, 1988. **10**(1): p. 33-46.
8. White, W.I., C.B. Evans, and D.W. Taylor, *Antimalarial antibodies of the immunoglobulin G2a isotype modulate parasitemias in mice infected with Plasmodium yoelii.* Infect Immun, 1991. **59**(10): p. 3547-54.
9. Shear, H.L., C. Ng, and Y. Zhao, *Cytokine production in lethal and non-lethal murine malaria.* Immunol Lett, 1990. **25**(1-3): p. 123-7.
10. Zhang, M., et al., *Macrophages expressing heat-shock protein 65 play an essential role in protection of mice infected with Plasmodium yoelii.* Immunology, 1999. **97**(4): p. 611-5.
11. Moneriz, C., et al., *Parasitostatic effect of maslinic acid. II. Survival increase and immune protection in lethal Plasmodium yoelii-infected mice.* Malar J, 2011. **10**: p. 103.
12. Mouray, E., et al., *Biochemical properties and cellular localization of Plasmodium falciparum protein disulfide isomerase.* Biochimie, 2007. **89**(3): p. 337-46.
13. Kicska, G.A., et al., *Effect of dietary p-aminobenzoic acid on murine Plasmodium yoelii infection.* J Infect Dis, 2003. **188**(11): p. 1776-81.
14. Pritzker, L.B., M. Scatena, and C.M. Giachelli, *The role of osteoprotegerin and tumor necrosis factor-related apoptosis-inducing ligand in human microvascular endothelial cell survival.* Mol Biol Cell, 2004. **15**(6): p. 2834-41.
15. Holder, A.A. and R.R. Freeman, *Immunization against blood-stage rodent malaria using purified parasite antigens.* Nature, 1981. **294**(5839): p. 361-4.

16. Neuhoff, V., et al., *Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250.* Electrophoresis, 1988. **9**(6): p. 255-62.

17. Mendez, D., et al., *Combined proteomic approaches for the identification of specific amino acid residues modified by 4-hydroxy-2-nonenal under physiological conditions.* J Proteome Res, 2010. **9**(11): p. 5770-81.

18. Friesen, J., et al., *Natural immunization against malaria: causal prophylaxis with antibiotics.* Sci Transl Med, 2010. **2**(40): p. 40ra49.

19. Good, M.F., *A whole parasite vaccine to control the blood stages of Plasmodium: the case for lateral thinking.* Trends Parasitol, 2011. **27**(8): p. 335-40.

20. Roestenberg, M., et al., *Protection against a malaria challenge by sporozoite inoculation.* N Engl J Med, 2009. **361**(5): p. 468-77.

21. Roestenberg, M., et al., *Long-term protection against malaria after experimental sporozoite inoculation: an open-label follow-up study.* Lancet, 2011. **377**(9779): p. 1770-6.

22. Mahajan, B., et al., *Multiple antigen peptide vaccines against Plasmodium falciparum malaria.* Infect Immun, 2010. **78**(11): p. 4613-24.

23. Trieu, A., et al., *Sterile protective immunity to malaria is associated with a panel of novel P. falciparum antigens.* Mol Cell Proteomics, 2011. **10**(9): p. M111 007948.

24. Alaro, J.R., M.M. Lynch, and J.M. Burns, Jr., *Protective immune responses elicited by immunization with a chimeric blood-stage malaria vaccine persist but are not boosted by Plasmodium yoelii challenge infection.* Vaccine, 2010. **28**(42): p. 6876-84.

25. Doolan, D.L., et al., *Profiling humoral immune responses to P. falciparum infection with protein microarrays.* Proteomics, 2008. **8**(22): p. 4680-94.

26. Bergmann-Leitner, E.S., et al., *Evaluation of immunoglobulin purification methods and their impact on quality and yield of antigen-specific antibodies.* Malar J, 2008. **7**: p. 129.

27. Qoronfleh, M.W., et al., *Use of Immunomatrix Methods to Improve Protein-Protein Interaction Detection.* J Biomed Biotechnol, 2003. **2003**(5): p. 291-298.

28. Ren, L., et al., *Improved immunomatrix methods to detect protein:protein interactions.* J Biochem Biophys Methods, 2003. **57**(2): p. 143-57.

29. Roque, A.C., C.S. Silva, and M.A. Taipa, *Affinity-based methodologies and ligands for antibody purification: advances and perspectives.* J Chromatogr A, 2007. **1160**(1-2): p. 44-55.

30. Borden, P. and E.A. Kabat, *An immunochemical analysis of precipitating and non-precipitating idiotype-anti-idiotype reactions.* Mol Immunol, 1990. **27**(6): p. 487-94.

31. Kaboord, B. and M. Perr, *Isolation of proteins and protein complexes by immunoprecipitation.* Methods Mol Biol, 2008. **424**: p. 349-64.

32. Crabb, B.S., T.F. de Koning-Ward, and P.R. Gilson, *Protein export in Plasmodium parasites: from the endoplasmic reticulum to the vacuolar export machine.* Int J Parasitol, 2010. **40**(5): p. 509-13.

33. de Koning-Ward, T.F., et al., *A newly discovered protein export machine in malaria parasites.* Nature, 2009. **459**(7249): p. 945-9.

34. Snowden, C.J., et al., *In vivo analysis of the lumenal binding protein (BiP) reveals multiple functions of its ATPase domain.* Plant J, 2007. **52**(6): p. 987-1000.

35. Novo, C., et al., *Gene sequencing, modelling and immunolocalization of the protein disulfide isomerase from Plasmodium chabaudi.* Int J Biol Macromol, 2009. **45**(4): p. 399-406.

36. Mahajan, B., et al., *Protein disulfide isomerase assisted protein folding in malaria parasites.* Int J Parasitol, 2006. **36**(9): p. 1037-48.

37. Yadava, A. and C.F. Ockenhouse, *Effect of codon optimization on expression levels of a functionally folded malaria vaccine candidate in prokaryotic and eukaryotic expression systems.* Infect Immun, 2003. **71**(9): p. 4961-9.

38. Tolia, N.H., et al., *Structural basis for the EBA-175 erythrocyte invasion pathway of the malaria parasite Plasmodium falciparum.* Cell, 2005. **122**(2): p. 183-93.

39. Guevara Patino, J.A., et al., *Antibodies that inhibit malaria merozoite surface protein-1 processing and erythrocyte invasion are blocked by naturally acquired human antibodies.* J Exp Med, 1997. **186**(10): p. 1689-99.

40. Dutta, S., et al., *Purification, characterization, and immunogenicity of the refolded ectodomain of the Plasmodium falciparum apical membrane antigen 1 expressed in Escherichia coli.* Infect Immun, 2002. **70**(6): p. 3101-10.

41. Baruch, D.I., et al., *Immunization of Aotus monkeys with a functional domain of the Plasmodium falciparum variant antigen induces protection against a lethal parasite line.* Proc Natl Acad Sci U S A, 2002. **99**(6): p. 3860-5.

42. Behr, C., et al., *Antibodies and reactive T cells against the malaria heat-shock protein Pf72/Hsp70-1 and derived peptides in individuals continuously exposed to Plasmodium falciparum.* J Immunol, 1992. **149**(10): p. 3321-30.

43. Biswas, S. and Y.D. Sharma, *Lack of correlation between red cell invasion by merozoites and anti-heat shock protein-70 antibody levels in malaria patients' sera.* Int J Parasitol, 1991. **21**(2): p. 213-7.

44. Kumar, N., et al., *Human immune response directed against Plasmodium falciparum heat shock-related proteins.* Infect Immun, 1990. **58**(5): p. 1408-14.

45. Renia, L., et al., *A malaria heat-shock-like determinant expressed on the infected hepatocyte surface is the target of antibody-dependent cell-mediated cytotoxic mechanisms by nonparenchymal liver cells.* Eur J Immunol, 1990. **20**(7): p. 1445-9.

46. Fontaine, A., et al., *Specific antibody responses against membrane proteins of erythrocytes infected by Plasmodium falciparum of individuals briefly exposed to malaria.* Malar J, 2010. **9**: p. 276.

47. Crompton, P.D., et al., *A prospective analysis of the Ab response to Plasmodium falciparum before and after a malaria season by protein microarray.* Proc Natl Acad Sci U S A, 2010. **107**(15): p. 6958-63.

48. Coombs, G.H., et al., *Aspartic proteases of Plasmodium falciparum and other parasitic protozoa as drug targets.* Trends Parasitol, 2001. **17**(11): p. 532-7.

49. Dame, J.B., et al., *Plasmepsin 4, the food vacuole aspartic proteinase found in all Plasmodium spp. infecting man.* Mol Biochem Parasitol, 2003. **130**(1): p. 1-12.

50. Omara-Opyene, A.L., et al., *Genetic disruption of the Plasmodium falciparum digestive vacuole plasmepsins demonstrates their functional redundancy.* J Biol Chem, 2004. **279**(52): p. 54088-96.

51. Liu, J., et al., *The role of Plasmodium falciparum food vacuole plasmepsins.* J Biol Chem, 2005. **280**(2): p. 1432-7.

52. Spaccapelo, R., et al., *Plasmepsin 4-deficient Plasmodium berghei are virulence attenuated and induce protective immunity against experimental malaria.* Am J Pathol, 2010. **176**(1): p. 205-17.

53. Omodeo-Sale, F., et al., *Dysregulation of L-arginine metabolism and bioavailability associated to free plasma heme.* Am J Physiol Cell Physiol, 2010. **299**(1): p. C148-54.

54. Yeo, T.W., et al., *Impaired nitric oxide bioavailability and L-arginine reversible endothelial dysfunction in adults with falciparum malaria.* J Exp Med, 2007. **204**(11): p. 2693-704.

55. Betney, R., et al., *Autoregulatory systems controlling translation factor expression: thermostat-like control of translational accuracy.* RNA, 2010. **16**(4): p. 655-63.

56. Daly, T.M. and C.A. Long, *Humoral response to a carboxyl-terminal region of the merozoite surface protein-1 plays a predominant role in controlling blood-stage infection in rodent malaria.* J Immunol, 1995. **155**(1): p. 236-43.

57. John, C.C., et al., *Evidence that invasion-inhibitory antibodies specific for the 19-kDa fragment of merozoite surface protein-1 (MSP-1 19) can play a protective role against blood-stage Plasmodium falciparum infection in individuals in a malaria endemic area of Africa.* J Immunol, 2004. **173**(1): p. 666-72.

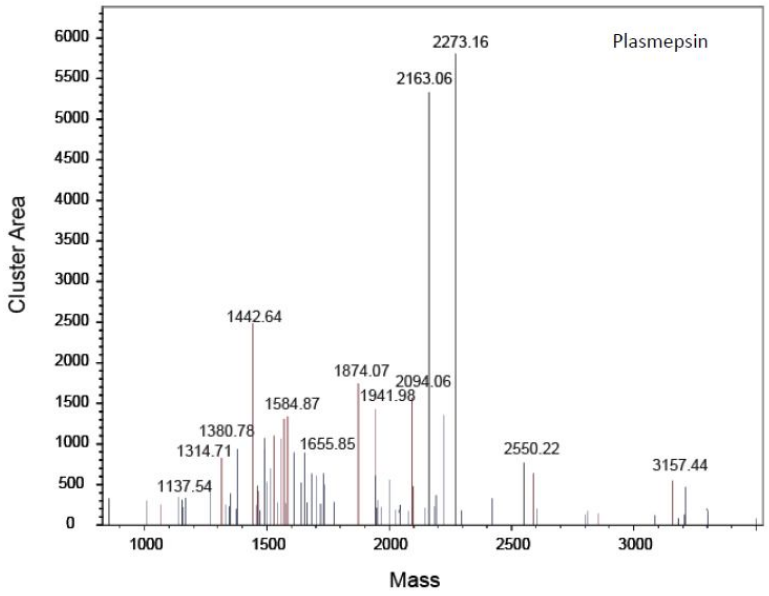
58. Blackman, M.J. and A.A. Holder, *Secondary processing of the Plasmodium falciparum merozoite surface protein-1 (MSP1) by a calcium-dependent membrane-bound serine protease: shedding of MSP133 as a noncovalently associated complex with other fragments of the MSP1.* Mol Biochem Parasitol, 1992. **50**(2): p. 307-15.

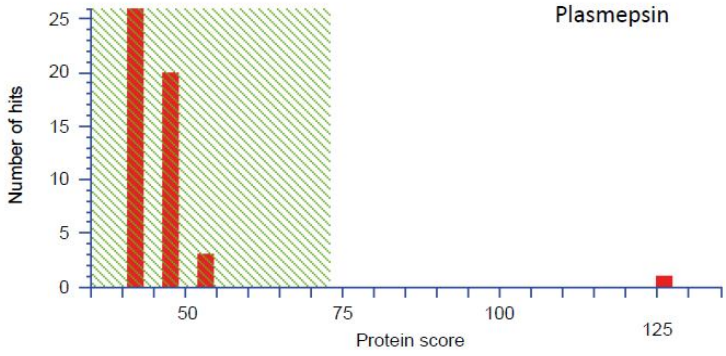
59. Ellis, R.D., et al., *Blood stage vaccines for Plasmodium falciparum: current status and the way forward.* Hum Vaccin, 2010. **6**(8): p. 627-34.

60. Goodman, A.L. and S.J. Draper, *Blood-stage malaria vaccines - recent progress and future challenges.* Ann Trop Med Parasitol, 2010. **104**(3): p. 189-211.

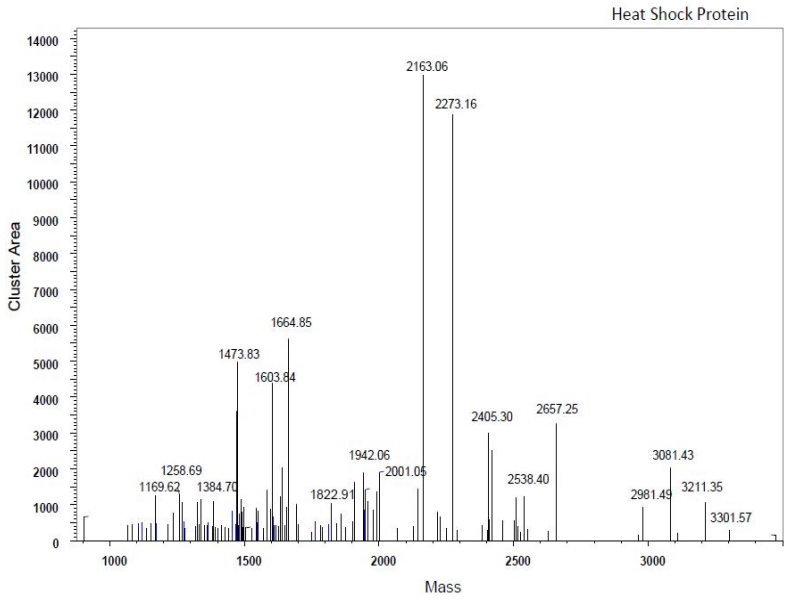
SUPPLEMENTARY MATERIAL

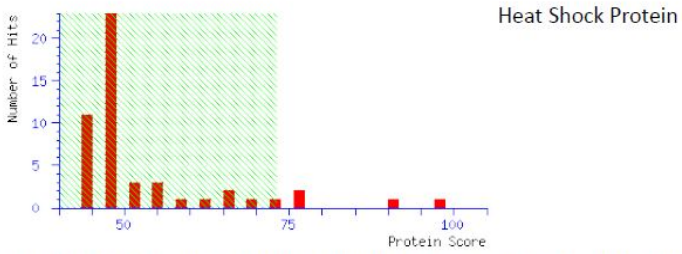
Fig. S1. MS analysis of trypsin-digested peptides, distribution of MOWSE score by Mascot search and total and matched sequences (bold) of the identified PY17XL proteins: plasmepsin, heat shock protein, protein disulfide isomerase, 39kDa subunit of eukaryotic translation initiation factor 3; and mice proteins: plasminogen, beta-2-glycoprotein 1 and fibrinogen beta and gamma chains. Scores higher than 73 were considered significant (P<0.05). Matched sequences of the identified protein are shown in bold.





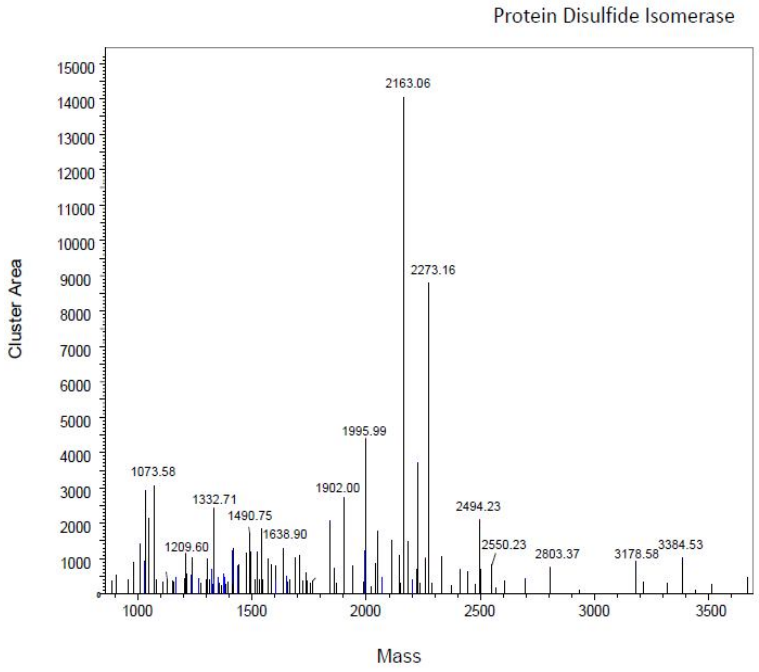
1 MAFKNYIYIY IFKMEYSEKE SNYSNGLMRN GSAFGQLKFD NIKSFKIQKK
51 FQALYFIIFV CIIGSIFAYL VGSNYYSTKT TDINKIIANS EYLTIRTKID
101 RPRDKLLKKV MNQNVSNYIK ESFRLLKSGL LKKEHLSKYD DGIELEQTIG
151 LAFFGTASLG DNKQSFTFIL DTGSSNLWVP NTDCKSGGCP YKHRYDSSTS
201 HTYEK**DGTPV SILYGSGGIK GFFSNDIFTI GHHTIPYKFI EVTQTDDLEP**
251 **IYTASEFDGI IGLGWKSLAV GNVEPVIVEM KKRGQIENAV FSFYLPEAEK**
301 **SIGYFTIGGI EESFYTGDLT YEK**LTNESYW QINLDVAFGI VTLDNANIIV
351 DSGTSAITAP SDFLEK**FLNT IMSIPVPFLP LRIVLCDDRN LPTLKFTSKN**
401 **TTYTIEPKHY LLELDPMAEI CAVAIVDVDI DPKTFILGDV FFKKYYTVFD**
451 **YDNSR**VGFAL AKN

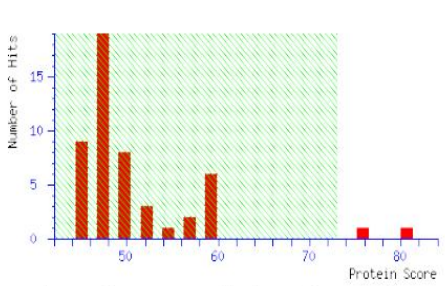




Distribution of MOWSE score for detected peptides by Mascot search for heat shock protein

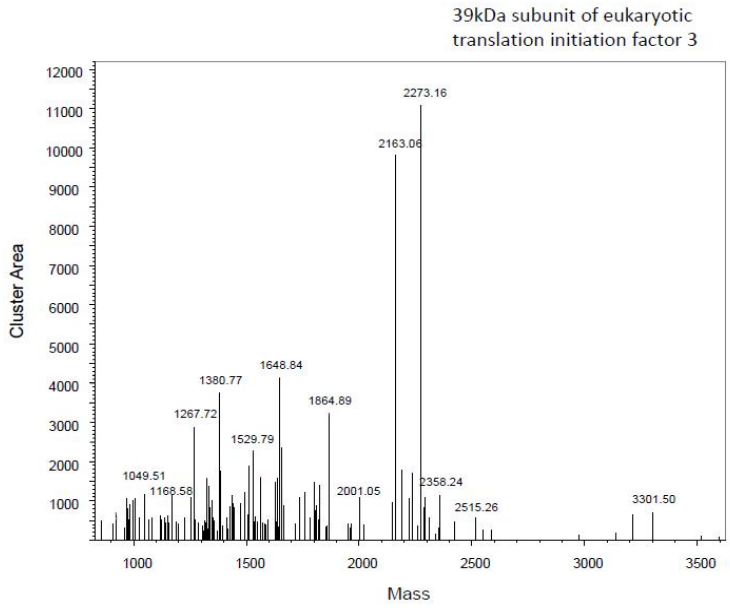
1 MGNTKAFVLV LLVSLLKFVS AVDSAKYTYI YIWCGFGLNI SSSIYMCKYV
51 FLFSIFSTYF SFSYFLYFFI FLFSIFSHFP IFLVEGPIIG IDLGTTYSCV
101 GVFK**NGRVEI LNNDLGNRIT PSYVSFVDGE R**KVGEAAK**LE ATLHPTQTVF**
151 DVKRLIGRKF DDKEVAKDR**T LLPYEIVNNE GKPNIK**VQIK DKPTTFAPEQ
201 ISAMVLEKMK EIAQSFLGKP VK**NAVVTVPA YFNDAQRQAT KDAGAIAGLN**
251 IVRIINEPTA AALAYGLDKK EETSILVYDL GGGTFDVSIL VIDNGVFEVY
301 ATAGNTHLGG EDFDQRVMDY FIKMFKKKNN IDLRSDKRAI QKLRKEVEIA
351 KR**NLSVVHST QIEIEDIIEG HNFSETLTRA KFEELNDDLF R**ETLEPVKKV
401 LDDAKYEK**SK IDEIVLVGGS TR**IPKIQQII KDFFNGKEPN RGINPDEAVA
451 YGAAIQAGII LGEELQDVVL LDVTPLTLGI ETVGGIMTQL IKRNTVIPTK
501 KSQTFSTYQD NQPAVLIQVF EGERALTK**DN HLLGKFELSG IPPAQR**GVPK
551 IEVTFTVDKN GILHVEAEDK GTGKSKGITI TNDKGRLSKE QIEKMINDAE
601 KFADEDKNLR EKVESKNNLD NYIQSMKATV EDKDKLADKI EKEDKDTILN
651 AIKEAEDWLN NNSNADSEAL KQKLK**DVEAI CQPIIVK**LYG QPGAASPPPG
701 DEDVDSDEL

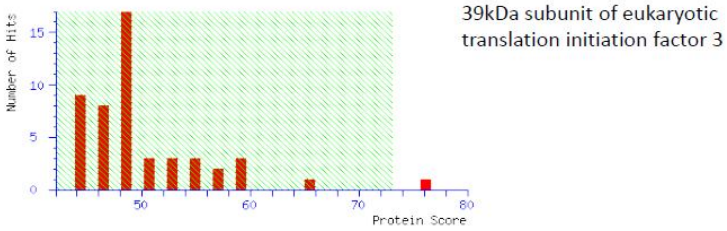




Distribution of MOWSE score for detected peptides by Mascot search for protein disulfide isomerase

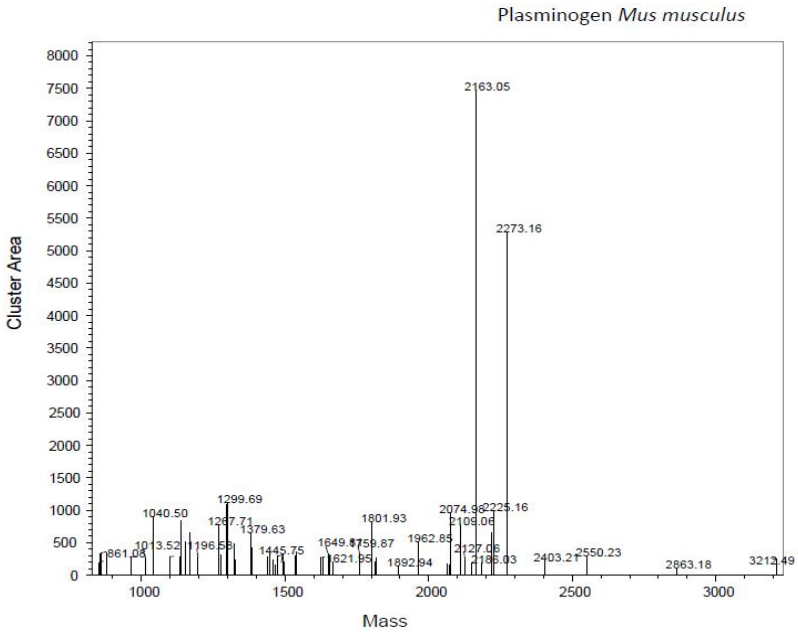
1 GVYLNKAPKM NTKYISFLLF LIPFVFKNYV RSHEDLFNEH ITSIHDGELS
51 NFITKNDIVL VMFYAPWCGH CKR**LIPEYNE AAIMLSEK**KS EIKLASVDAT
101 IER**GLSQEYG ITGYPTMILF NKKN**RINYGG GRTAQTIVDW ILQMTGPVST
151 EITGNIEDVL KEKNINVAFY MEYTSEDNEL FK**MFNEVGDK NR**EIAKYFMK
201 KNDKHNKIYC YRKDEKTVEY DEKTPLNDFV SIESFPLFGE INTENYR**FYA**
251 ESPKELVWVC ATVEQYNEIK EEVRLAAAEL R**NKTHFVLLN IPEYADHAKA**
301 SLGINEFPGL AYQSSEGRYL LTNPQQSLKN HKDIISFFKD VEAGKIEKSL
351 KSEPIPEEDK NAAVKVVVGN SFIDVVLNSG KDVLIEIYAP WCGHCKK**LEP**
401 VYEELGRKLK KYDHIIVAKM DGTLNETALK EFEWSGFPTI FFVK**AGSKIP**
451 LPYEGERTLK GFVDFLNKHS TKTPITIDGV SQSDEGSSEE L

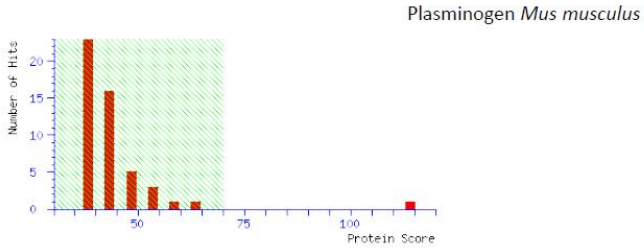




Distribution of MOWSE score for detected peptides by Mascot search for eukaryotic translation initiation factor 3, 39kDa subunit

1 MKRKYLCGHN RPLTHVNTNC DGDLLFTTGR DKKFILWNLA DGNQIGLYEC
51 SGAVYNSDVT FDSKRVVCSS AANK**IYIFDV YTGETLK**VID ESGPVR**FVEF**
101 NRDPLNQNRV IAAIDRLKAD HKRFIKLYDL K**SDTLIWKQE HESR**CIQVRW
151 CFFDKLILSA HENGEIVIWN SEDGHQIRKF QAHTK**EVTNL SFDKDRMIML**
201 SSSTDGTAIL RDAVNFDIIN EYKTDRPLNT CDISPLFRSE NNPKNHIILA
251 GGQAAEHVTT TATGEGKFQT LLYDIIHANE LGSIK**GHFGT VHSIKFLPHG**
301 DGFVSGGEDG FARIYHFDQD YFIGKYD

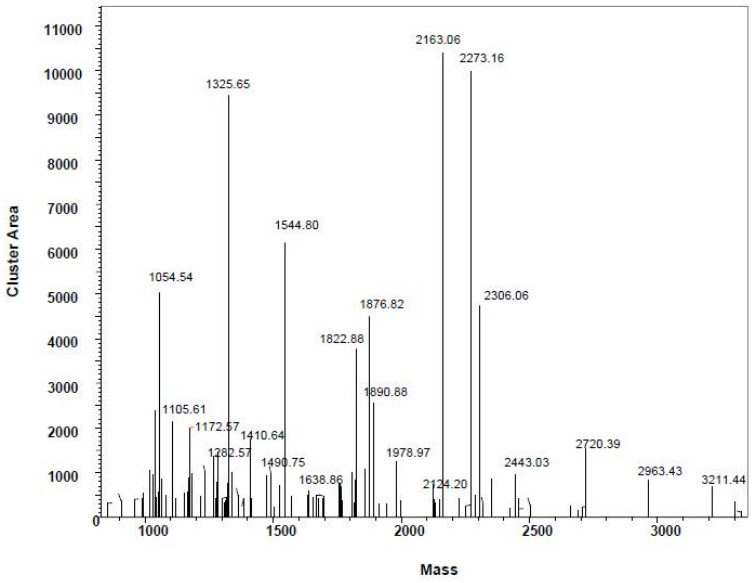




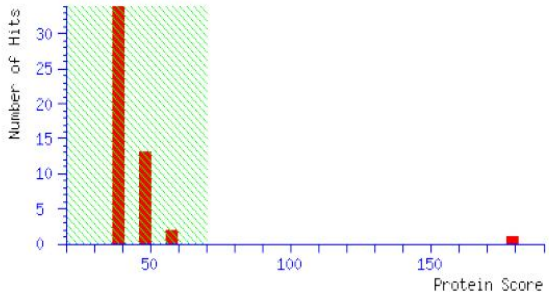
Distribution of MOWSE score for detected peptides by Mascot search for mice plasminogen.

1 MDHKEVILLF LLLLKPGQGD SLDGYISTQG ASLFSLTKKQ LAAGGVSDCL
51 AKCEGETDFV CRSFQYHSKE QQCVIMAENS KTSSIIRMRD VILFEKRVYL
101 SECKTGIGNG YRGTMSRTKS GVACQKWGAT FPHVPNYSPS THPNEGLEEN
151 YCRNPDNDEQ GPWCYTTDPD KRYDYCNIPE CEEECMYCSG EKYEGKISKT
201 MSGLDCQAWD SQSPHAHGYI PAKFPSKNLK MNYCRNPDGE PRPWCFTTDP
251 TKR**WEYCDIP R**CTTPPPPPS PTYQCLKGRG ENYRGTVSVT VSGKTCQR**WS**
301 EQTPHRHNRT PENFPCK**NLE ENYCRNPDGE TAPWCYTTDS QLR**WEYCEIP
351 SCESSASPDQ SDSSVPPEEQ TPVVQECYQS DGQSYRGTSS TTITGKK**CQS**
401 WAAMFPHRHS KTPENFPDAG LEMNYCR**NPD GDKGPWCYTT DPSVRWEYCN**
451 LKRCSETGGS VVELPTVSQE PSGPSDSETD CMYGNGKDYR GK**TAVTAAGT**
501 PCQGWAAQEP HRHSIFTPQT NPRAGLEKNY CR**NPDGDVNG PWCYTTNPR**K
551 LYDYCDIPLC ASASSFECGK PQVEPKKCPG RVVGGCVANP HSWPWQISLR
601 TRFTGQHFCG GTLIAPEWVL TAAHCLEK**SS RPEFYKVILG AHEEYIR**GSD
651 VQEISVAK**LI LEPNNRDIAL LK**LSRPATIT DK**VIPACLPS PNYMVADRTI**
701 CYITGWGETQ GTFGAGRLKE AQLPVIENKV CNRVEYLNNR VKSTELCAGQ
751 LAGGVDSCQG DSGGPLVCFE KDKYILQGVT SWGLGCARPN KPGVYVRVSR
801 FVDWIEREMR NN

Beta-2-glycogen *Mus musculus*



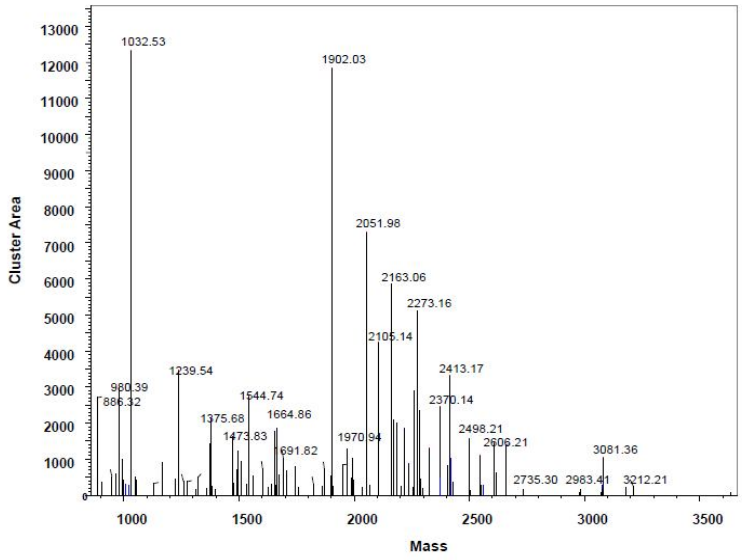
Beta-2-glycoportein *Mus musculus*



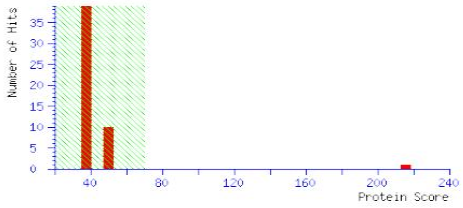
Distribution of MOWSE score for detected peptides by Mascot search for mice beta-2-glycoprotein.

1 MVSPVLALFS AFLCHVAIAG RICPKPDDLP FATVVPLK**TS YDPGEQIVYS**
51 CKPGYVSRGG MR**RFTCPLTG MWPINTLR**CV PR**VCPFAGIL ENGIVR**YTSF
101 EYPKNISFAC NPGFFLNGTS SSK**CTEEGKW SPDIPACARI TCPPPPVPK**F
151 ALLKDYRPSA GNNSLYQDTV VFKCLPHFAM IGNDTVMCTE QGNWTRLPEC
201 LEVKCPFPPR PENGYVNYPA KPVLLYKDK**A TFGCHETYKL DGPEEAECTK**
251 TGTWSFLPTC RESCKLPVK**K ATVLYQGMRV KIQEQFK**NGM MHGDKIHFYC
301 KNKEK**KCSYT VEAHCRDGTI EIPSCFKEHS SLAFWK**TDAS ELTPC

Fibrinogen beta chain *Mus musculus*



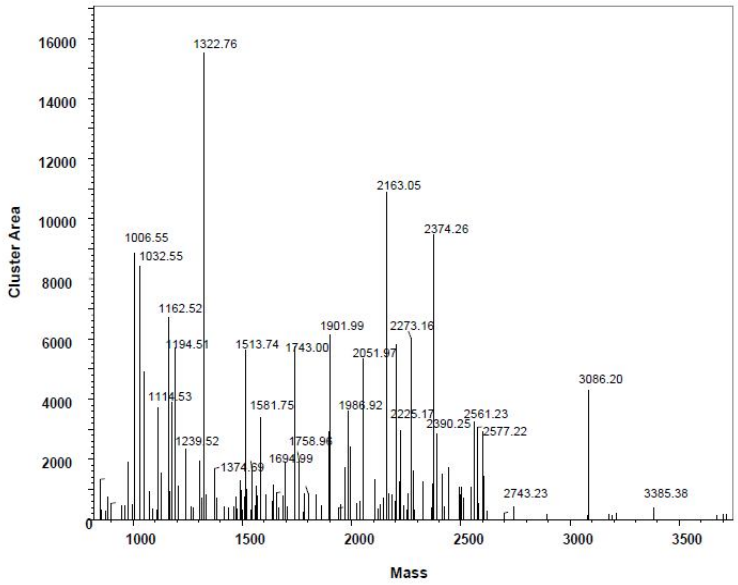
Fibrinogen beta-chain *Mus musculus*



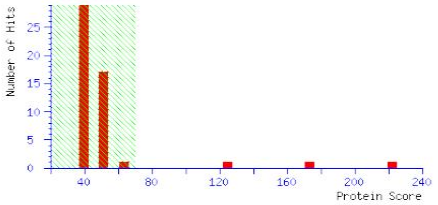
Distribution of MOWSE score for detected peptides by Mascot search for mice fibrinogen beta chain.

1 MRHLWLLLLL CVFSVQTQAA DDDYDEPTDS LDARGHRPVD RR**KEEPPSLR**
51 PAPPPISGGG YRARPAKATA NQKKVERRPP DAGGCLHADT DMGVLCPTGC
101 TLQQTLLNQE RPIKSSIAEL NNNIQSVSDT SSVTFQYLTL LKDMWKKK**QA**
151 QVKENENVIN EYSSILEDQR LYIDETVNDN IPLNLRVLRS ILEDLRSK**IQ**
201 KLESDISAQM EYCRTPCTVS CNIPVVSGKE CEEIIRKGGE TSEMYLIQPD
251 TSIKPYRVYC DMKTENGGWT VIQNRQDGSV DFGRKWDPYK KGFGNIATNE
301 DAKK**YCGLPG EYWLGNDKIS QLTRMGPTEL LIEMEDWK**GD KVK**AHYGGFT**
351 VQNEASKYQV SVNKYKGTAG NALMDGASQL VGENRTMTIH NGMFFSTYDR
401 DNDGWVTTDP RK**QCSKEDGG GWWYNR**CHAA NPNGR**YYWGG LYSWDMSKHG**
451 TDDGVVWMNW KGSWYSMRR**M SMKIRPFFPQ Q**

Fibrinogen gamma-chain *Mus musculus*



Fibrinogen gamma-chain *Mus musculus*



Distribution of MOWSE score for detected peptides by Mascot search for mice fibrinogen gamma chain.

1 MSWSLQPPSF LLCCLLLLFS PTGLAYVATR **DNCCILDERF GSFCPTTCGI**
51 ADFLSSYQTD VDNDLRTLED ILFRAENR**TT EAKELIKAIQ VYYNPDQPPK**
101 PGMIDSATQK SKK**MVEEIVK YEALLLTHET SIRYLQEIYN SNNQK**ITNLK
151 QKVAQLEAQC QEPCKDSVQI HDTTGKDCQE IANKGAK**ESG LYFIRPLKAK**
201 QQFLVYCEID GSGNGWTVLQ KRIDGSLDFK KNWIQYK**EGF GHLSPTGTTE**
251 FWLGNEKIHL ISMQSTIPYA LRIQLKDWNG RTSTADYAMF RVGPESDKYR
301 LTYAYFIGGD AGDAFDGYDF GDDPSDKFFT SHNGMQFSTW DNDNDKFEGN
351 CAEQDGSGWW MNKCHAGHLN GVYHQGGTYS K**SSTTNGFDD GIIWATWK**SR
401 WYSMKETTMK IIPFNR**LSIG EGQQHHMGGS KQAGDV**

4.2 CHAPTER 2

Partial immunity to lethal rodent malaria elicited by immunoaffinity-purified blood-stage parasite antigens

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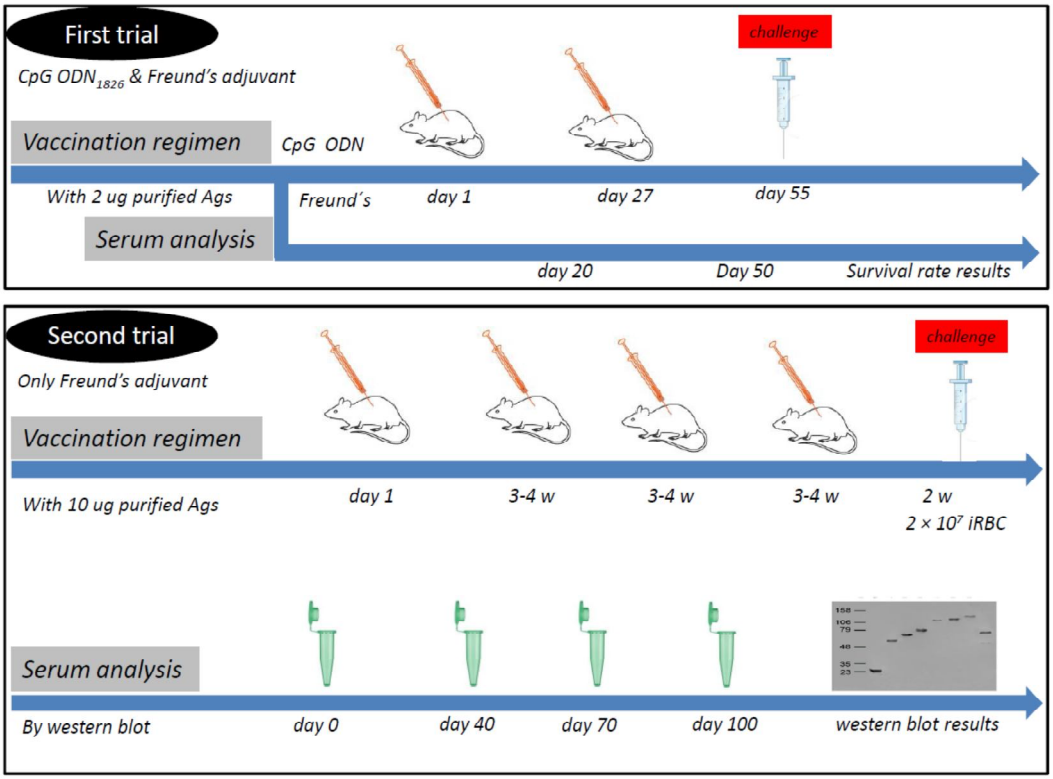
GRAPHICAL ABSTRACT



IgGs from malaria resistant mice sera to lethal doses infection of *Plasmodium yoelii yoelii* 17XL, were purified and subsequently immobilized for isolation of blood-stage parasite antigens. Immunoaffinity purified antigens were used to immunize BALC/c mice using CpG ODN_{1826} and Freund's adjuvant system. Vaccinated mice serum at different days post vaccination was analyzed by western blot. Parasitemia and survival rate were also monitored after challenging infection.

Abstract

An effective malaria subunit vaccine remains elusive despite intensive efforts. Here a lethal malaria murine model was used to assess multiple affinity purified blood-stage parasite antigens for the ability to induce immunity to blood stage malaria. For this study, we have generated malaria-resistant ICR mice to lethal doses of *Plasmodium yoelii yoelii* 17XL whose sera was used for purification of their infection-induced IgGs. Immobilized IgGs were subsequently used to isolate blood-stage parasite antigens. These immunoaffinity purified antigens were used to immunize BALC/c mice. Western blot analysis of vaccinated mice serum at different days post vaccination showed an intensive immune response to large number of antigens with molecular weight ranging between 22 to 250 kDa. The level of protection obtained in vaccinated BALB/c mice after challenging to high lethal doses of *P. yoelii yoelii* 17XL was heterogeneous. Nonetheless, this immunization with multiple antigens isolated by immunoaffinity from intact native parasites induced a delayed infection of the erythrocyte resulting in a partial protection against malaria disease. This approach shows the potential to prevent malaria with a set of antigens isolated from blood-stage parasites.

Keywords

Malaria; Antigens; Adjuvants; Subunit vaccine; Immunity.

4.3 CHAPTER 3

Posttranslational carbonylation of *Plasmodium yoelii* antigens identified by immunoproteomics with malaria-resistant mice sera

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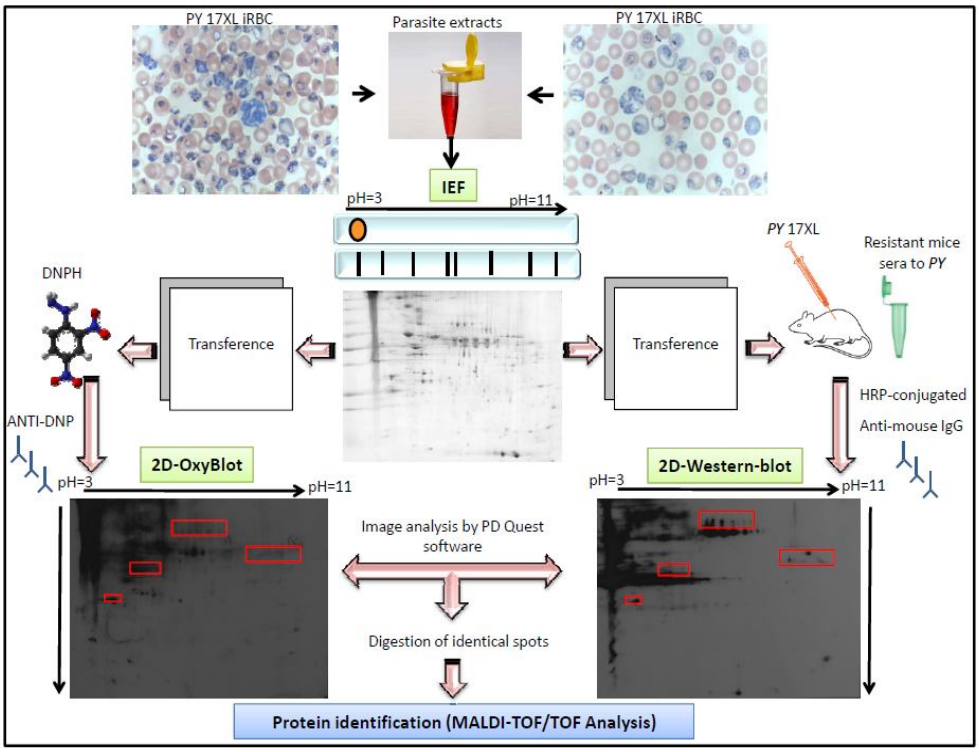
GRAPHICAL ABSTRACT



Taking advantage of post-electrophoretic DNPH derivatization of oxidized protein and using ICR mice sera resistant to lethal infection of *Plasmodium yoelii* 17XL, generated in our laboratory, several potential vaccine antigens were identically identified by both immunochemical and immunoproteomic methods during blood-stage life cycle of Plasmodium parasite.

Abstract

The evidences from partial naturally acquired protective immunity to malaria infection in some individuals, in malaria endemic areas, have raised hopes to development of effective vaccines against malaria. Beside the elusiveness of immunological mechanisms involved in naturally acquired immunity, studies on malaria vaccine development suggest that the malaria immunity which lead to protection is highly dependent on the immune response against several parasite proteins which large number of them still remain unidentified. Hence, identification and analysis of immuno-protective parasite antigens would eventually results in the development of improved formulations for vaccine development. On the other hand, during blood-stage infection the parasite induce a high oxidative stress which oxidatively modify the proteins in the red cell environment, including host and parasite proteins. Herein, taking advantage of post-electrophoretic DNPH derivatization of oxidized protein, and using ICR mice sera resistant to lethal infection of *Plasmodium yoelii* 17XL generated in our laboratory, we have comparatively identified several potential antigens identically by both immunochemical and immunoproteomic methods, during the blood-stage life cycle of Plasmodium parasite. Beside identification of known potential target vaccine antigens like merozoite surface protein 1 and merozoite surface antigen PY230, our results shown 6 fate protein newly identified: heat shock protein 90, heat shock protein ClpB, co-chaperon GrpE, Cpn20 protein, heat shock protein 70 and protein disulfide isomerase, these last two recently identified by immunoproteomic analysis. Notably, 2 out of 6 fate protein (co-chaperon GrpE and Cpn20 protein) were identified for the first time and likewise 5 novel blood-stage plasmodial metabolic enzyme (adenosine deaminase, ATP synthase F1 subunit beta, dihydrolipoamide dehydrogenase and FAD-dependent glycerol-3-phosphate dehydrogenase and mitochondrial processing peptidase subunit alpha homolog) were additionally identified. Here, the hypothetical roles of the newly identified antigens as potential stimulator host immune response, and those already known vaccine target antigens, are discussed in details. Taking together, our finding with brief proposed hypothetical roles of those discovered novel antigens, may help to design further

5 General discussion

In the present study, regarding the results obtained from protein A/G spin column and IP kit (Chapter one), the Ig purification method was evaluated on the basis of purity, antibody specificity and functionality using resistance mice serum to malaria. When taking into consideration, both yield and purity, we concluded that the highest quality Ig could be achieved using protein A/G, also the optimal Ig purification methods relative to high-level Ag-specific Ig recovery and functionality for resistance mice serum. This is in agreement with methodology employed by other authors where rabbit and human Igs were also purified preferentially by protein A/G [236-238].

Immobilized bacterial surface proteins that interact with the Fc portion of IgG (Protein A—SpA, and Protein G—SpG) or with immunoglobulin light chains (Protein L—PpL) are the most frequent affinity ligands for the purification of antibodies and genetically derived molecules (Figure 12) [160]. The binding properties of different bacterial proteins with respect to different antibodies from several mammalian species is given in Table 5.

Table 5. Properties of the immunoglobulin-binding bacterial proteins A, G and L (+++ very strong binding; ++strong binding; + moderate binding; - no binding).

Immunoglobulin		Protein L	Protein A	Protein G
Human Ig	IgG	+++	+++	+++
	IgM, IgA, IgE,	++	+	-
	IgD	++	+	+
	Fab, F(ab`)$_2$, scFv	+++	+	-
Mouse Ig	IgG$_1$, IgG$_3$	++	+	++
	IgG$_{2a}$, IgG$_{2b}$	++	++	++
	IgM	++	+	-
	IgA	++	++	+
Polyclonal	Mouse	++	++	++
	Rat	++	+	++
	Rabbit	+	++	+++
	Goat, Bovine	-	+	++
	Porcine	++	+	+

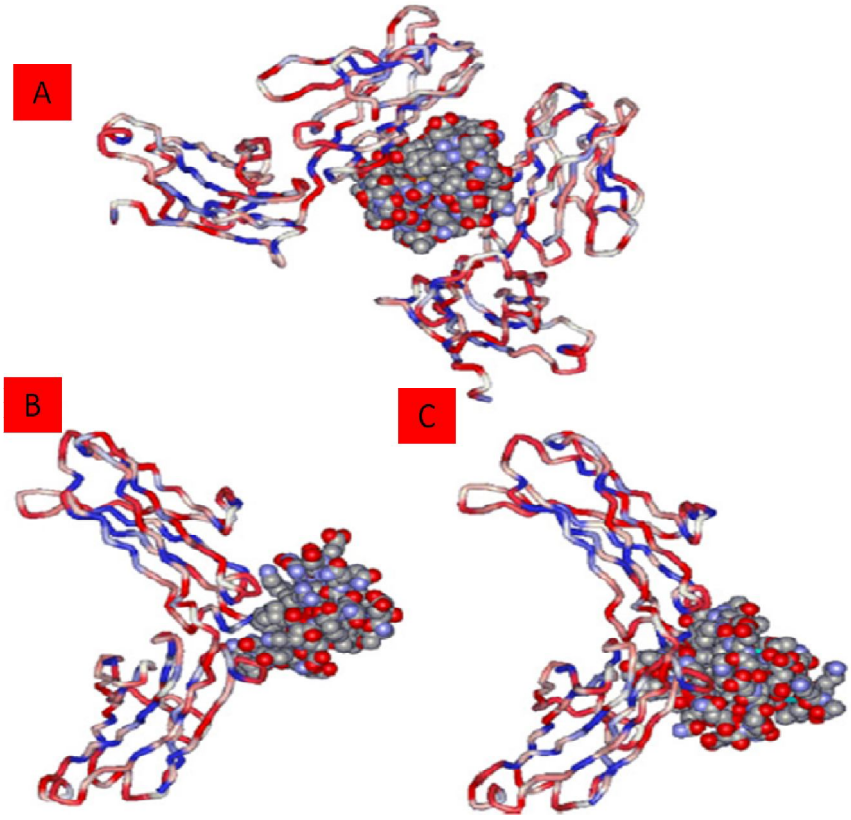


Figure 12. (A) A PpL single domain binds simultaneously the VL region of two IgG Fab fragments, through a β-zipper interaction (PDB code: 1HEZ). (B) Fragment B of Protein A binds to the Fc domain of IgG (PDB code: 1FC2). (C) Protein G domain binding the Fc portion of IgG (PDB code: 1FCC). Taken from [230].

Moreover, according to the immunobloting follow up of the purification, no change in Ab affinity for Igs were observed. The ability to purify Ig from sera or plasma from immune animals or humans with a high yield and without selective loss of isotypes is imperative when conducting analytical experiments [236].

Apart from the *in vitro* analysis of humoral immune response, Igs are frequently batch-purified from serum for the purpose of passive transfer of the Ig as therapeutic agents into naive animals and even human host. In either case, it is imperative that the purification method dose not introduce artifacts, such as a bias in the Ig isotype, or alter the functional activity of the antibody molecules.

From the results mentioned above and those that observed in chapter one, the procedure of purifying, isolating and identifying parasite antigens from serum IgGs of malaria-protected individuals are in especial interest for further study in the field of

immune response and vaccine developing approach and could be a novel strategy for the development of multi-antigen based vaccine therapies. Further, the identification of 4 plasmodial proteins in Plamodium yoelii blood-stage infection in chapter one, will help us to understand more in details in the biology of Plasmodium parasite.

Although improved vaccine technologies have been central to attempts to develop more-effective pre-erythrocytic-stage vaccines (Figure 13) studies of asexual blood stages during the past 20 years have revealed a detailed array of molecules associated with parasite development and pathogenesis, and the natural acquisition of immunity [239].

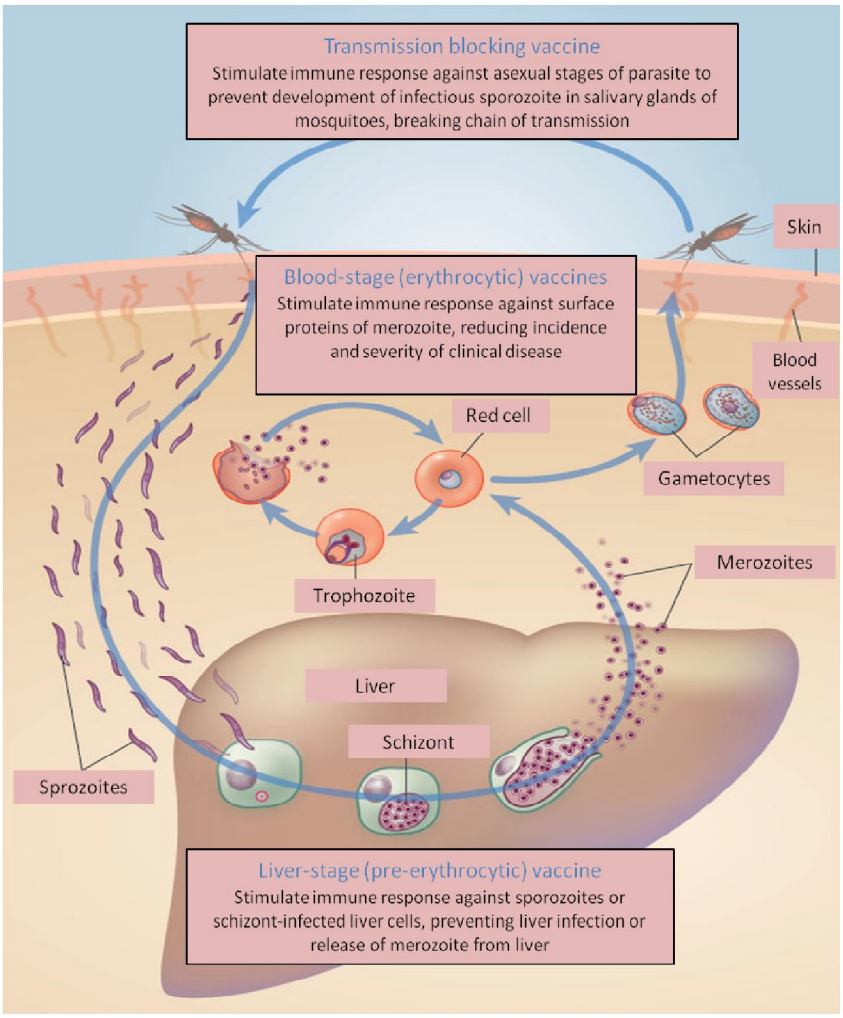


Figure 13. Vaccine Targets in Malaria
Liver-stage vaccines are designed to prevent malaria infection, but they must be 100 percent effective to protect people with no natural immunity (such as soldiers and travelers). They include vaccines containing whole killed sporozoites and those based on antigenic portions of sporozoite proteins. Most blood-stage vaccines seek to elicit antibodies to merozoites (the blood-cell–infecting stage of the parasite), since in people with natural immunity, such antibodies are associated with protection from clinical illness. Variability of blood-stage antigens among parasite strains has complicated vaccine development. Transmission-blocking vaccines would not protect the recipient but could help to prevent the spread of malaria. Sexual-stage parasite antigens are complex and have been difficult to produce. Multistage vaccines target antigens from multiple stages of the parasite's life cycle. Some malaria researchers believe such a cocktail may be necessary for high efficacy, but these vaccines are complicated and expensive to make.

At the molecular level, the advanced have been remarkable during this time and are set to continue with the genomic and proteomic data that are accumulating [240, 241]. That said, the asexual-blood-stage target antigen that is undergoing the most intensive investigation is still, as in 1985, the merozoite surface protein MSP-1. It was already recognized in 1985 that the asexual blood stage are the major target of

naturally acquired immune responses, that immunization with merozoite antigens involved red blood cell invasion is likely to be complicated by antigenic diversity and that the preferred molecules or epitopes for inclusion in a vaccine might, consequently, be those that are nonvariant [242]. Naturally acquired immunity in a highly endemic setting is a state of premonition. Individuals remain immune and asymptomatic because they have low-grade chronic infections [242]. This acquired immunity is induced predominantly by antigens that are polymorphic or that undergo clonal antigenic variation [14]. This contributes to the chronic state of infection by enabling the parasite to evade immune response. In one of the few clinical trials carried out with asexual-blood-stage antigens, vaccination with MSP-1, MSP-2 and the ring-infected erythrocyte surface antigen (RESA) reduced parasite density significantly, but this was a strain-specific effect [140].

Apical membrane antigen (AMA)-1, another vaccine candidate that has been known since 1985, is also highly polymorphic and, similary, induces strain-specific immunity [99]. Despite the (AMA)-1 is one of the top vaccine candidates because it can effectively inhibit the invasion of merozoites into red blood cells, this antigen has a low priority in vaccines considered for advanced clinical development because its antigenic diversity compromise vaccine efficacy [243]. However, it has been shown that chimaeras of two different antigens induce inhibition of two malaria strains [244]. In this case a comprehensive approach to map the immunodominant epitopes in different variants may help the design of novel molecules able to elicit a broad immune response. Additional efforts to broaden the response to AMA1 have been recently reported [245, 246]. The ability to engineer successfully antigens able to induce broad immune responses using structure based design of immunodominant epitopes has been shown in a recent work where the meningococcus antigen factor H binding protein, which is present in three different variants, was engineered to induce protective antibodies against all natural variants of the antigen [247].

A fair conclusion that is drawn frequently from studies of natural immunity is that, for vaccine development, it would be better to focus on cryptic epitopes [240] rather than epitopes of the highly immunogenic polymorphic or clonally variant domains [248]. However, some promising liver- and blood-stage candidate vaccine

molecules have been selected after analysis of naturally acquired immune response [249, 250]. Also, the variant surface antigens (VSAs) of parasites that cause severe disease are different from and more immunogenic than those isolated from cases of mild malaria [251]. The possibility of exploiting this as a vaccine strategy should be also taken into consideration. Some of the candidate antigens under investigation are poorly immunogenic because they have a limited number of T-cell determinants; hence, they are MHC restricted and induce an immune response in only subsets of the population. There are ways to overcome this that have been known for a long time, notably coupling the relatively small vaccine molecules to carriers containing T-cell epitops [252]. Other features of the asexual blood stage of infection that could compromise the induction of a strong response to vaccines are that parasitized erythrocytes can suppress maturation of dendritic cells, thus impairing antigen presentation to T cells [238], and can cause apoptosis of malaria-specific T cells and B cells [242]. The emphasis on developing candidate asexual-blood-stage vaccines continues to be based on subunit strategies but, stimulated by studies from more than 20 years ago, malaria-naive volunteers were recently shown to be fully protected against homologous challenge if immunized using extremely low-dose infections by inoculation of ~30 erythrocytes infected with *P.falciparum* on three occasions, with each infection being drug cured eight days after infection. Of particular interest is the observation that protection seemed to be cell mediated rather than antibody mediated [253]. This has the appearance of an innate, cytokine-mediated protection induced early in the infection [12]. However, this re-awakening of the whole organism approach to vaccination against blood stages requires further investigation to see whether there is a feasible way of exploiting it, perhaps by focusing on antigens that are targets of cell-mediated immunity [254]. However, the findings from the early studies using whole parasite approaches can now be viewed with a better understanding of the mechanisms of immunity, the characteristics of naturally acquired immunity to malaria [13], and explored in detail because of the major advances in both laboratory research capacity and biotechnology. Indeed, revising the early studies of protective immunity induced by radiation attenuated sporozoites [117] has led to one the most innovative advances in malaria vaccine research, namely the program by Hoffman and colleagues to develop a pre-erythrocytic vaccine using

radiation attenuated sporozoites. A brief summary of previous studies in rodents of whole killed and attenuated blood stage vaccines are presented in the following tables.

Table 6. Mouse studies of blood stage immunization by infection-cure.

Host species	Vaccine	Challenge	Protection	Notes	Ref
Mouse (CFI white)	*P.chabaudi* iRBC 10^6	10^7 *P. Vinckei* 10^6 *P.berghei*	84% protection 0% protection		[255]
Mouse (CH3)	*P. vinkei*; drug cure with chloroquine	*P. Vinckei* *P.berghei*	100% 0%	Killed parasites not protective; splenectomy attenuated immunity	[256]
Mouse (Swiss)	*P. chabaudi*; drug cure with chloroquine	*P.berghei*	Delayed mortality		[257]
Mouse	*P. Vinckei; P. Chabaudi; P.berghei; P.yoelii*	Cross protection study	All resistant to homologous challenge; cross-challenge protection varied with species		[258]
Mouse (B-cell deficient)	*P. Vinckei; P. Chabaudi; P.b.yoelii; drug cure with chloroquine as required*	Cross protection study	Varied with species; improved survival in most cross-protection studies.		[259]
Mouse CBA/Ca	*P.berghei; P. Chabaudi AS, P.chabaudi DS*	Cross protection study	All resistant to homologous challenge; cross-challenge protection varied with species		[260]
Mouse CBA/Ca	*P. Chabaudi* AS, *P. Yoelii* 17XL	Cross protection study	*P. chabaudi* AS with natural cure protected against homologous and heterologous challenge. Effect of drug cure on subsequend development of immunity depended on the timing of drug administration	Early drug cure prevented development of protection to homologous and heterologous parasites.	[261]
C57BL/6j mice	10^5 *P. chabaudi* x 3 doses with drug cure	10^6 iRBC (homologous & heterologous strains)	Attenuation of parasitemia; heterologous protection		[262]
Mouse (BALB/c)	*P. yoelii* 10^5 iRBC with drug cure x 1	*P. yoelii* 10^5 iRBC	100% protection	Protection against sporozoite challenge. B cells required for protection against blood stage	[263]
Mouse (BALB/c)	*P. yoelii* (256BY nonlethal) drug cure with chloroquine	*P. yoelii* (256BY & 17X non-lethal strain)	100%		[264]

Table 7. Mouse studies of blood stage immunization with blood stage parasite fractions.

Host species	Vaccine	Challenge	Protection	Ref
Mouse (AJ)	*P. berghei* lysate x 1	*P. berghei*	100% protection with crude lysate and high level protection with certain fractions.	[265]
Mouse	*P. chabaudi* merozoites (10^4–10^6) in FCA x 2	*P. chabaudi* iRBC (10^6–10^8)	50% survival in lethal (10^6) challenge following 10^6 x 2 vaccinations	[266]
Mouse	*P. berghei*, *P. vinckei*, *P. chabaudi*, *P. yoelii*	Cross protection study	All resistant to homologous challenge; crosschallenge protection varied with species	[258]
Mouse (AJ)	*P. chabaudi* 10^7 iRBC + Alum + IL-12 x 2 doses (2^{nd} dose no IL-12) or 10^7 iRBC + Alum + CpG x 2 doses	10^6 iRBC	100% protection from lethal challenge	[267]

Table 8. Rodent studies of blood stage immunization with irradiated/attenuated parasites.

Host species	Vaccine	Challenge	Protection	Notes	Ref
Mouse (albino)	Irradiated *P. berghei*; immunizations with irradiated iRBC (1 x 10^8) x 5 doses		significantly delayed mortality, with 5/35 (14% survival)		[268]
Rat	Irradiated *P. berghei*		significant attenuation in challenge parasitemia following single or multiple doses of irradiated iRBCs	Non-lethal model;	[268]
Mouse (ICR)	Irradiated *P. berghei*; immunizations with irradiated iRBC (1 x 10^8) x 5 doses		significantly delayed mortality, with 6/10 surviving low dose challenge	survival related to challenge dose	[269]
Mouse (ICR)	Irradiated *P. yoelii*	*P. yoelii* sporozoites	Attenuated parasitemia		[269]
Mice (BALB/c)	*P. berghei* 10^7 radiation-attenuated parasites	*P. berghei* 10^6	7/11 Protected against lethal challenge		[270]
Mice (BALB/c)	*P. yoelii* 5 x 10^6 radiation-attenuated lines	*P. yoelii* 10^4	5/5 Protected against lethal challenge		[271]
Mouse (BALB/c)	Attenuated *P. yoelii*	*P. yoelii* (YM lethal strain	100%		[272]

The growing realization of the likely limitations of recombinant protein-based malaria vaccines, coupled with a better understanding of the protective immunity to

malaria, both in animal models and in naturally exposed human populations and experimentally infected volunteers, as well as the increased capacity to manipulate parasites provides new impetus to evaluate whole blood stage parasite approaches to malaria vaccine development. Progress made in pre-erythrocytic radiation attenuated parasites as malaria vaccine candidates and recent advances in constructing stable genetically attenuated parasites provide further support to this. While it appears that so-called sterile immunity may be a difficult goal to achieve with a blood stage whole parasite vaccine, recent clinical experimental studies [253, 273] indicate that robust protective immunity may be a realistic goal and the anti-disease protection that such a vaccine would confer would complement the efficacy of any pre-erythrocytic vaccine which, if less than 100% efficacious would still make populations vulnerable to the potentially lethal outcome of breakthrough blood stage infection. It is likely that any blood stage parasite vaccine using whole killed parasite material will need to be formulated and delivered in a way to elicit a protective cellular immune response. In this respect, the selection of a potent and safe adjuvant will be a critical step [156]. Safety issues such as red cell alloimmunization and adventitious infectious agents will need to be addressed. An alternate approach is the use of live but genetically attenuated parasites. There is much optimism for this approach for pre-erythrocytic vaccines, and there is emerging data on the potential utility of parasites with stable genetic attenuation [272]. However, in addition to the biosafety issues referred to above, an ability to cryopreserve parasites or another technical solution for vaccine delivery will be required.

Effective vaccines for malaria must reproduce or, even better, improve naturally acquired immunity. However, the latter, which is directed primarily against asexual blood stages, requires repeated exposure and involves persistence of infection, responses to complex antigenic polymorphisms, immune modulation and immune evasion. On the basis, it has been argued that, to be effective, a vaccine should not induce a sterilizing immunity, certainly against the clinically important phase of infection [274]. The goal for pre-erythrocytic (and transmission-blocking) vaccines remains the prevention of all parasite development, but this is far from being achieved at presents. It is doubtful whether any of the vaccines currently scheduled for clinical trials will, on their own, have the efficacy and long-term effectiveness to justify

widescale use. This will probably be achieved only with combination, multi-component vaccines.

A vaccine that is good enough to be an effective alternative to treatment and vector control remains a more distant goal and might require another 20 years to perfect [239].

CONCLUSIONS

5.1 CONCLUSIONS

The experimental results presented in this thesis provide new insights into malaria multi-antigen-based vaccine therapies and the redox biology of the malaria parasite. According to our results we can raise the following conclusions:

1- A protein A/G system allowed the purification of different subclasses of IgGs from malaria-immunized mice. The results obtained with the A/G system demonstrated that the high-affinity purified IgGs retain their structure and functionality throughout the purification processes.

2- The combined approach of protein A/G immunoglobulin purification, antigen enrichment by immunoaffinity using immobilized immunoglobulins and the identification of the obtained proteins by mass spectrometry was used for the search of novel potential antigens in blood-stage *Plasmodium yoelii* lethal malaria infection allowing the identification of four peptides: protein disulfide isomerase, a member of the heat shock protein 70 family, plasmepsin and a 39 kDa-subunit of the eukaryotic translation initiation factor 3. One or more of these proteins may be suitable for further testing as components of vaccine formulations.

3- Freund´s adjuvant system and subcutaneous route of inoculation were appropriate approaches to stimulate mice immune system, allowing the isolation of multiple antigens by immunoaffinity.

4- The combined set of antigens obtained after purification by immunoafinity of *P. yoelii* proteins triggered a good immune response in vaccinated mice, however, infection of mice with lethal doses of *P.yoelii* 17XL resulted only in partial protection against malaria disease, showing lower parasitemia profiles and delayed death as compared to untreated controls.

5- Six *P. yoelii* 17XL carbonylated proteins recovered from infected mice erythrocytes were identified by immunoblots and MS analysis as novel immunogenic candidates: co-chaperon GrpE, Cpn20 protein, FAD-dependent glycerol-3-phosphate dehydrogenase, dihydrolipoamide dehydrogenase, ATP synthase F1 subunit beta and adenosine deaminase. These proteins may be involved in essential maturation and metabolic processes during the

erythrocytic cycle of Plasmodium, and either alone or in combination with the previously identified immunogenic proteins may be used in model systems to test candidate vaccines.

REFERENCES

6 REFERENCES

1. Breman, J.G., *The ears of the hippopotamus: manifestations, determinants, and estimates of the malaria burden.* Am J Trop Med Hyg, 2001. **64**(1-2 Suppl): p. 1-11.
2. Wipasa, J., et al., *Immunity to asexual blood stage malaria and vaccine approaches.* Immunol Cell Biol, 2002. **80**(5): p. 401-14.
3. Prudencio, M., A. Rodriguez, and M.M. Mota, *The silent path to thousands of merozoites: the Plasmodium liver stage.* Nat Rev Microbiol, 2006. **4**(11): p. 849-56.
4. Bannister, L.H., et al., *A brief illustrated guide to the ultrastructure of Plasmodium falciparum asexual blood stages.* Parasitol Today, 2000. **16**(10): p. 427-33.
5. Miller, L.H., et al., *The pathogenic basis of malaria.* Nature, 2002. **415**(6872): p. 673-9.
6. Engwerda, C.R. and M.F. Good, *Disarming the malaria parasite.* Nat Med, 2008. **14**(9): p. 912-3.
7. Hall, N., et al., *A comprehensive survey of the Plasmodium life cycle by genomic, transcriptomic, and proteomic analyses.* Science, 2005. **307**(5706): p. 82-6.
8. Hernandez-Valladares, M., J. Naessens, and F.A. Iraqi, *Genetic resistance to malaria in mouse models.* Trends Parasitol, 2005. **21**(8): p. 352-5.
9. Li, C., E. Seixas, and J. Langhorne, *Rodent malarias: the mouse as a model for understanding immune responses and pathology induced by the erythrocytic stages of the parasite.* Med Microbiol Immunol, 2001. **189**(3): p. 115-26.
10. Marsh, K., *Malaria - a neglected disease.* Parasitology, 1992. **104**: p. S53-S69.
11. Baird, J.K., *Host age as a determinant of naturally acquired immunity to Plasmodium falciparum.* Parasitology Today, 1995. **11**(3): p. 105-111.
12. Stevenson, M.M. and E.M. Riley, *Innate immunity to malaria.* Nat Rev Immunol, 2004. **4**(3): p. 169-80.
13. Doolan, D.L., C. Dobano, and J.K. Baird, *Acquired immunity to malaria.* Clin Microbiol Rev, 2009. **22**(1): p. 13-36, Table of Contents.
14. Bull, P.C., et al., *Parasite antigens on the infected red cell surface are targets for naturally acquired immunity to malaria.* Nat Med, 1998. **4**(3): p. 358-60.
15. Riley, E.M., et al., *Naturally acquired cellular and humoral immune responses to the major merozoite surface antigen (PfmSP1) of Plasmodium falciparum are associated with reduced malaria morbidity.* Parasite Immunology, 1992. **14**(3): p. 321-337.
16. Hogh, B., et al., *Relattionship between maternally derived anti-Plasmodium falciparum antibodies and risk of infection and disease in infants living in an area of Liberia, west-Africa, in which malaria is highly endemic.* Infection and Immunity, 1995. **63**(10): p. 4034-4038.
17. AlYaman, F., et al., *Assessment of the role of naturally acquired antibody levels to Plasmodium falciparum merozoite surface protein-1 in protecting Papua New Guinean children from malaria morbidity.* American Journal of Tropical Medicine and Hygiene, 1996. **54**(5): p. 443-448.
18. Egan, A.F., et al., *Clinical immunity to Plasmodium falciparum malaria is associated with serum antibodies to the 19-kDa C-terminal fragment of the merozoite surface antigen, PfMSP-1.* Journal of Infectious Diseases, 1996. **173**(3): p. 765-769.

19. Branch, O.H., et al., *A longitudinal investigation of IgG and IgM antibody responses to the merozoite surface protein-1 19-kilodalton domain of Plasmodium falciparum in pregnant women and infants: Associations with febrile illness, parasitemia, and anemia.* American Journal of Tropical Medicine and Hygiene, 1998. **58**(2): p. 211-219.

20. Dodoo, D., et al., *Levels of antibody to conserved parts of Plasmodium falciparum merozoite surface protein 1 in Ghanaian children are not associated with protection from clinical malaria.* Infection and Immunity, 1999. **67**(5): p. 2131-2137.

21. Conway, D.J., et al., *A principal target of human immunity to malaria identified by molecular population genetic and immunological analyses.* Nature Medicine, 2000. **6**(6): p. 689-692.

22. Cavanagh, D.R., et al., *Antibodies to the N-terminal block 2 of Plasmodium falciparum merozoite surface protein 1 are associated with protection against clinical malaria.* Infection and Immunity, 2004. **72**(11): p. 6492-6502.

23. Perraut, R., et al., *Antibodies to the conserved C-terminal domain of the Plasmodium falciparum merozoite surface protein 1 and to the merozoite extract and their relationship with in vitro inhibitory antibodies and protection against clinical malaria in a Senegalese village.* Journal of Infectious Diseases, 2005. **191**(2): p. 264-271.

24. Bouharoun-Tayoun, H., et al., *Antibodies that protect humans against Plasmodium falciparum blood stages do not on their own inhibit parasite growth and invasion in vitro, but act in cooperation with monocytes.* J Exp Med, 1990. **172**(6): p. 1633-41.

25. Narum, D.L., et al., *Immunization with parasite-derived apical membrane antigen 1 or passive immunization with a specific monoclonal antibody protects BALB/c mice against lethal Plasmodium yoelii yoelii YM blood-stage infection.* Infect Immun, 2000. **68**(5): p. 2899-906.

26. Spencer Valero, L.M., et al., *Passive immunization with antibodies against three distinct epitopes on Plasmodium yoelii merozoite surface protein 1 suppresses parasitemia.* Infect Immun, 1998. **66**(8): p. 3925-30.

27. Langhorne, J., et al., *A role for B cells in the development of T cell helper function in a malaria infection in mice.* Proc Natl Acad Sci U S A, 1998. **95**(4): p. 1730-4.

28. von der Weid, T., N. Honarvar, and J. Langhorne, *Gene-targeted mice lacking B cells are unable to eliminate a blood stage malaria infection.* J Immunol, 1996. **156**(7): p. 2510-6.

29. Piper, K.P., et al., *Malaria transmission and naturally acquired immunity to PfEMP-1.* Infect Immun, 1999. **67**(12): p. 6369-74.

30. Egan, A.F., M.J. Blackman, and D.C. Kaslow, *Vaccine efficacy of recombinant Plasmodium falciparum merozoite surface protein 1 in malaria-naive, -exposed, and/or -rechallenged Aotus vociferans monkeys.* Infect Immun, 2000. **68**(3): p. 1418-27.

31. Hirunpetcharat, C., et al., *Complete protective immunity induced in mice by immunization with the 19-kilodalton carboxyl-terminal fragment of the merozoite surface protein-1 (MSP1[19]) of Plasmodium yoelii expressed in Saccharomyces cerevisiae: correlation of protection with antigen-specific antibody titer, but not with effector CD4+ T cells.* J Immunol, 1997. **159**(7): p. 3400-11.

32. Hirunpetcharat, C., et al., *Absolute requirement for an active immune response involving B cells and Th cells in immunity to Plasmodium yoelii passively acquired with antibodies to the 19-kDa carboxyl-terminal fragment of merozoite surface protein-1.* J Immunol, 1999. **162**(12): p. 7309-14.

33. Aucan, C., et al., *Familial correlation of immunoglobulin G subclass responses to Plasmodium falciparum antigens in Burkina Faso.* Infect Immun, 2001. **69**(2): p. 996-1001.

34. Aribot, G., et al., *Pattern of immunoglobulin isotype response to Plasmodium falciparum blood-stage antigens in individuals living in a holoendemic area of Senegal (Dielmo, west Africa).* Am J Trop Med Hyg, 1996. **54**(5): p. 449-57.

35. Bouharoun-Tayoun, H. and P. Druilhe, *Plasmodium falciparum malaria: evidence for an isotype imbalance which may be responsible for delayed acquisition of protective immunity.* Infect Immun, 1992. **60**(4): p. 1473-81.

36. Aucan, C., et al., *High immunoglobulin G2 (IgG2) and low IgG4 levels are associated with human resistance to Plasmodium falciparum malaria.* Infect Immun, 2000. **68**(3): p. 1252-8.

37. Jayawardena, A.N., et al., *The immunological response of CBA mice to P. yoelii. I. General characteristics, the effects of T-cell deprivation and reconstitution with thymus grafts.* Immunology, 1977. **32**(6): p. 849-59.

38. Jayawardena, A.N., et al., *The immunological response of CBA mice to P. yoelii. II. The passive transfer of immunity with serum and cells.* Immunology, 1978. **34**(1): p. 157-65.

39. Freeman, R.R. and C.R. Parish, *Plasmodium yoelii: antibody and the maintenance of immunity in BALB/c mice.* Exp Parasitol, 1981. **52**(1): p. 18-24.

40. Langhorne, J., et al., *Immunoglobulin isotype distribution of malaria-specific antibodies produced during infection with Plasmodium chabaudi adami and Plasmodium yoelii.* Cell Immunol, 1984. **87**(2): p. 452-61.

41. Matsumoto, S., et al., *Mycobacterium bovis bacillus calmette-guerin induces protective immunity against infection by Plasmodium yoelii at blood-stage depending on shifting immunity toward Th1 type and inducing protective IgG2a after the parasite infection.* Vaccine, 2000. **19**(7-8): p. 779-87.

42. Smith, N.C., et al., *The spleen, IgG antibody subsets and immunity to Plasmodium berghei in rats.* Immunol Cell Biol, 1997. **75**(3): p. 318-23.

43. Taylor-Robinson, A.W. and R.S. Phillips, *B cells are required for the switch from Th1- to Th2-regulated immune responses to Plasmodium chabaudi chabaudi infection.* Infect Immun, 1994. **62**(6): p. 2490-8.

44. Majarian, W.R., et al., *Passive immunization against murine malaria with an IgG3 monoclonal antibody.* J Immunol, 1984. **132**(6): p. 3131-7.

45. Vukovic, P., et al., *Immunoglobulin G3 antibodies specific for the 19-kilodalton carboxyl-terminal fragment of Plasmodium yoelii merozoite surface protein 1 transfer protection to mice deficient in Fc-gammaRI receptors.* Infect Immun, 2000. **68**(5): p. 3019-22.

46. Bouharoun-Tayoun, H., et al., *Mechanisms underlying the monocyte-mediated antibody-dependent killing of Plasmodium falciparum asexual blood stages.* J Exp Med, 1995. **182**(2): p. 409-18.

47. Tebo, A.E., P.G. Kremsner, and A.J. Luty, *Plasmodium falciparum: a major role for IgG3 in antibody-dependent monocyte-mediated cellular inhibition of parasite growth in vitro.* Exp Parasitol, 2001. **98**(1): p. 20-8.

48. Perraut, R., et al., *Induction of opsonizing antibodies after injection of recombinant Plasmodium falciparum vaccine candidate antigens in preimmune Saimiri sciureus monkeys.* Infect Immun, 1995. **63**(2): p. 554-62.

49. Giribaldi, G., et al., *Growth of Plasmodium falciparum induces stage-dependent haemichrome formation, oxidative aggregation of band 3, membrane deposition of complement and antibodies, and phagocytosis of parasitized erythrocytes.* Br J Haematol, 2001. **113**(2): p. 492-9.

50. Ramasamy, R. and R. Rajakaruna, *Association of malaria with inactivation of alpha1,3-galactosyl transferase in catarrhines.* Biochim Biophys Acta, 1997. **1360**(3): p. 241-6.

51. Ramasamy, R., M. Ramasamy, and S. Yasawardena, *Antibodies and Plasmodium falciparum merozoites.* Trends Parasitol, 2001. **17**(4): p. 194-7.

52. Ramasamy, R., et al., *Antibodies to a merozoite surface protein promote multiple invasion of red blood cells by malaria parasites.* Parasite Immunol, 1999. **21**(8): p. 397-407.

53. Guevara Patino, J.A., et al., *Antibodies that inhibit malaria merozoite surface protein-1 processing and erythrocyte invasion are blocked by naturally acquired human antibodies.* J Exp Med, 1997. **186**(10): p. 1689-99.

54. Abbas, A.K., K.M. Murphy, and A. Sher, *Functional diversity of helper T lymphocytes.* Nature, 1996. **383**(6603): p. 787-93.

55. Bienzle, U., et al., *Inhibition of Plasmodium vinckei-malaria in mice by recombinant murine interferon-gamma.* Acta Trop, 1988. **45**(3): p. 289-90.

56. Clark, I.A., et al., *Inhibition of murine malaria (Plasmodium chabaudi) in vivo by recombinant interferon-gamma or tumor necrosis factor, and its enhancement by butylated hydroxyanisole.* J Immunol, 1987. **139**(10): p. 3493-6.

57. Favre, N., et al., *The course of Plasmodium chabaudi chabaudi infections in interferon-gamma receptor deficient mice.* Parasite Immunol, 1997. **19**(8): p. 375-83.

58. Meding, S.J., et al., *Role of gamma interferon during infection with Plasmodium chabaudi chabaudi.* Infect Immun, 1990. **58**(11): p. 3671-8.

59. Shear, H.L., et al., *Role of IFN-gamma in lethal and nonlethal malaria in susceptible and resistant murine hosts.* J Immunol, 1989. **143**(6): p. 2038-44.

60. Stevenson, M.M., M.F. Tam, and M. Nowotarski, *Role of interferon-gamma and tumor necrosis factor in host resistance to Plasmodium chabaudi AS.* Immunol Lett, 1990. **25**(1-3): p. 115-21.

61. Watier, H., et al., *IFN-gamma treatment of rodents infected with erythrocytic stages of Plasmodium chabaudi: differential effects according to the immunological status.* Int J Immunopharmacol, 1993. **15**(3): p. 293-9.

62. Gazzinelli, R.T., *Molecular and cellular basis of interleukin 12 activity in prophylaxis and therapy against infectious diseases.* Mol Med Today, 1996. **2**(6): p. 258-67.

63. Sam, H. and M.M. Stevenson, *In vivo IL-12 production and IL-12 receptors beta1 and beta2 mRNA expression in the spleen are differentially up-regulated in resistant B6 and susceptible A/J mice during early blood-stage Plasmodium chabaudi AS malaria.* J Immunol, 1999. **162**(3): p. 1582-9.

64. Sam, H. and M.M. Stevenson, *Early IL-12 p70, but not p40, production by splenic macrophages correlates with host resistance to blood-stage Plasmodium chabaudi AS malaria.* Clin Exp Immunol, 1999. **117**(2): p. 343-9.

65. Yoshimoto, T., et al., *Interleukin-12-dependent mechanisms in the clearance of blood-stage murine malaria parasite Plasmodium berghei XAT, an attenuated variant of P. berghei NK65.* J Infect Dis, 1998. **177**(6): p. 1674-81.

66. Mohan, K., P. Moulin, and M.M. Stevenson, *Natural killer cell cytokine production, not cytotoxicity, contributes to resistance against blood-stage Plasmodium chabaudi AS infection.* J Immunol, 1997. **159**(10): p. 4990-8.

67. Choudhury, H.R., et al., *Early nonspecific immune responses and immunity to blood-stage nonlethal Plasmodium yoelii malaria.* Infect Immun, 2000. **68**(11): p. 6127-32.

68. Seder, R.A., et al., *Interleukin 12 acts directly on CD4+ T cells to enhance priming for interferon gamma production and diminishes interleukin 4 inhibition of such priming.* Proc Natl Acad Sci U S A, 1993. **90**(21): p. 10188-92.

69. Su, Z. and M.M. Stevenson, *Central role of endogenous gamma interferon in protective immunity against blood-stage Plasmodium chabaudi AS infection.* Infect Immun, 2000. **68**(8): p. 4399-406.

70. Yoneto, T., et al., *Gamma interferon production is critical for protective immunity to infection with blood-stage Plasmodium berghei XAT but neither NO production nor NK cell activation is critical.* Infect Immun, 1999. **67**(5): p. 2349-56.

71. Balmer, P., J. Alexander, and R.S. Phillips, *Protective immunity to erythrocytic Plasmodium chabaudi AS infection involves IFNgamma-mediated responses and a cellular infiltrate to the liver.* Parasitology, 2000. **121 Pt 5**: p. 473-82.

72. Luty, A.J., et al., *Low interleukin-12 activity in severe Plasmodium falciparum malaria.* Infect Immun, 2000. **68**(7): p. 3909-15.

73. Perkins, D.J., J.B. Weinberg, and P.G. Kremsner, *Reduced interleukin-12 and transforming growth factor-beta1 in severe childhood malaria: relationship of cytokine balance with disease severity.* J Infect Dis, 2000. **182**(3): p. 988-92.

74. Stevenson, M.M., et al., *IL-12-induced protection against blood-stage Plasmodium chabaudi AS requires IFN-gamma and TNF-alpha and occurs via a nitric oxide-dependent mechanism.* J Immunol, 1995. **155**(5): p. 2545-56.

75. Pichyangkul, S., P. Saengkrai, and H.K. Webster, *Plasmodium falciparum pigment induces monocytes to release high levels of tumor necrosis factor-alpha and interleukin-1 beta.* Am J Trop Med Hyg, 1994. **51**(4): p. 430-5.

76. Schofield, L. and F. Hackett, *Signal transduction in host cells by a glycosylphosphatidylinositol toxin of malaria parasites.* J Exp Med, 1993. **177**(1): p. 145-53.

77. Jacobs, P., D. Radzioch, and M.M. Stevenson, *A Th1-associated increase in tumor necrosis factor alpha expression in the spleen correlates with resistance to blood-stage malaria in mice.* Infect Immun, 1996. **64**(2): p. 535-41.

78. Butcher, G.A. and I.A. Clark, *The inhibition of Plasmodium falciparum growth in vitro by sera from mice infected with malaria or treated with TNF.* Parasitology, 1990. **101 Pt 3**: p. 321-6.

79. Haidaris, C.G., et al., *Serum containing tumor necrosis factor is cytotoxic for the human malaria parasite Plasmodium falciparum.* Infect Immun, 1983. **42**(1): p. 385-93.

80. Good, M.F. and D.L. Doolan, *Malaria vaccine design: immunological considerations.* Immunity, 2010. **33**(4): p. 555-66.

81. Eslava, I., et al., *Suppressive and additive effects in protection mediated by combinations of monoclonal antibodies specific for merozoite surface protein 1 of Plasmodium yoelii.* Malar J, 2010. **9**: p. 46.

82. Ma, C., et al., *Production, characterisation and immunogenicity of a plant-made Plasmodium antigen-the 19 kDa C-terminal fragment of Plasmodium yoelii merozoite surface protein 1.* Appl Microbiol Biotechnol, 2011.

83. Moss, D.K., et al., *Plasmodium falciparum merozoite surface protein (MSP) 119-specific antibodies that interfere with parasite growth in vitro can inhibit MSP1 processing, merozoite invasion and intracellular parasite development.* Infect Immun, 2011.

84. Shi, Q., et al., *Enhanced protection against malaria by a chimeric merozoite surface protein vaccine.* Infect Immun, 2007. **75**(3): p. 1349-58.

85. Cherif, M.S., et al., *Immunogenicity of novel nanoparticle-coated MSP-1 C-terminus malaria DNA vaccine using different routes of administration.* Vaccine, 2011. **29**(48): p. 9038-50.

86. Cowan, G.J., et al., *A malaria vaccine based on the polymorphic block 2 region of MSP-1 that elicits a broad serotype-spanning immune response.* PLoS One, 2011. **6**(10): p. e26616.

87. Holder, A.A., et al., *Processing of the precursor to the major merozoite surface antigens of Plasmodium falciparum.* Parasitology, 1987. **94 (Pt 2)**: p. 199-208.

88. Lyon, J.A., et al., *Epitope map and processing scheme for the 195,000-dalton surface glycoprotein of Plasmodium falciparum merozoites deduced from cloned overlapping segments of the gene.* Proc Natl Acad Sci U S A, 1986. **83**(9): p. 2989-93.

89. McBride, J.S. and H.G. Heidrich, *Fragments of the polymorphic Mr 185,000 glycoprotein from the surface of isolated Plasmodium falciparum merozoites form an antigenic complex.* Mol Biochem Parasitol, 1987. **23**(1): p. 71-84.

90. Blackman, M.J. and A.A. Holder, *Secondary processing of the Plasmodium falciparum merozoite surface protein-1 (MSP1) by a calcium-dependent membrane-bound serine protease: shedding of MSP133 as a noncovalently associated complex with other fragments of the MSP1.* Mol Biochem Parasitol, 1992. **50**(2): p. 307-15.

91. Egan, A.F., et al., *Human antibodies to the 19kDa C-terminal fragment of Plasmodium falciparum merozoite surface protein 1 inhibit parasite growth in vitro.* Parasite Immunol, 1999. **21**(3): p. 133-9.

92. Fowkes, F.J.I., et al., *The relationship between anti-merozoite antibodies and incidence of Plasmodium falciparum malaria: A systematic review and meta-analysis.* PLoS medicine, 2010. **7**(1): p. e1000218.

93. Holder, A.A. and R.R. Freeman, *Immunization against blood-stage rodent malaria using purified parasite antigens.* Nature, 1981. **294**(5839): p. 361-4.

94. Siddiqui, W.A., et al., *Merozoite surface coat precursor protein completely protects Aotus monkeys against Plasmodium falciparum malaria.* Proc Natl Acad Sci U S A, 1987. **84**(9): p. 3014-8.

95. Blackman, M.J., et al., *Antibodies inhibit the protease-mediated processing of a malaria merozoite surface protein.* J Exp Med, 1994. **180**(1): p. 389-93.

96. Tian, J.H., et al., *Comparison of protection induced by immunization with recombinant proteins from different regions of merozoite surface protein 1 of Plasmodium yoelii.* Infect Immun, 1997. **65**(8): p. 3032-6.

97. Mitchell, G.H., et al., *Apical membrane antigen 1, a major malaria vaccine candidate, mediates the close attachment of invasive merozoites to host red blood cells.* Infect Immun, 2004. **72**(1): p. 154-8.

98. Thomas, A.W., et al., *The Fab fragments of monoclonal IgG to a merozoite surface antigen inhibit Plasmodium knowlesi invasion of erythrocytes.* Mol Biochem Parasitol, 1984. **13**(2): p. 187-99.

99. Crewther, P.E., et al., *Protective immune responses to apical membrane antigen 1 of Plasmodium chabaudi involve recognition of strain-specific epitopes.* Infect Immun, 1996. **64**(8): p. 3310-7.

100. Xu, H., et al., *CD4+ T cells acting independently of antibody contribute to protective immunity to Plasmodium chabaudi infection after apical membrane antigen 1 immunization.* J Immunol, 2000. **165**(1): p. 389-96.

101. Collins, W.E., et al., *Protective immunity induced in squirrel monkeys with recombinant apical membrane antigen-1 of Plasmodium fragile.* Am J Trop Med Hyg, 1994. **51**(6): p. 711-9.

102. Kocken, C.H., et al., *High-level expression of Plasmodium vivax apical membrane antigen 1 (AMA-1) in Pichia pastoris: strong immunogenicity in Macaca mulatta immunized with P. vivax AMA-1 and adjuvant SBAS2.* Infect Immun, 1999. **67**(1): p. 43-9.

103. Fraser, T.S., et al., *Erythrocyte-binding activity of Plasmodium yoelii apical membrane antigen-1 expressed on the surface of transfected COS-7 cells.* Mol Biochem Parasitol, 2001. **117**(1): p. 49-59.

104. Freeman, R.R., A.J. Trejdosiewicz, and G.A. Cross, *Protective monoclonal antibodies recognising stage-specific merozoite antigens of a rodent malaria parasite.* Nature, 1980. **284**(5754): p. 366-8.

105. Borre, M.B., et al., *Multiple genes code for high-molecular-mass rhoptry proteins of Plasmodium yoelii.* Mol Biochem Parasitol, 1995. **70**(1-2): p. 149-55.

106. Narum, D.L., et al., *Sequence diversity and antigenic polymorphism in the Plasmodium yoelii p235 high molecular mass rhoptry proteins and their genes.* Mol Biochem Parasitol, 2001. **112**(2): p. 193-200.

107. Ogun, S.A. and A.A. Holder, *A high molecular mass Plasmodium yoelii rhoptry protein binds to erythrocytes.* Mol Biochem Parasitol, 1996. **76**(1-2): p. 321-4.

108. Ogun, S.A., et al., *A member of the py235 gene family of Plasmodium yoelii encodes an erythrocyte binding protein recognised by a protective monoclonal antibody.* Mol Biochem Parasitol, 2006. **147**(1): p. 140-3.

109. Galinski, M.R., et al., *A reticulocyte-binding protein complex of Plasmodium vivax merozoites.* Cell, 1992. **69**(7): p. 1213-26.

110. Rayner, J.C., et al., *Two Plasmodium falciparum genes express merozoite proteins that are related to Plasmodium vivax and Plasmodium yoelii adhesive proteins involved in host cell selection and invasion.* Proc Natl Acad Sci U S A, 2000. **97**(17): p. 9648-53.

111. Taylor, H.M., M. Grainger, and A.A. Holder, *Variation in the expression of a Plasmodium falciparum protein family implicated in erythrocyte invasion.* Infect Immun, 2002. **70**(10): p. 5779-89.

112. Triglia, T., et al., *Identification of proteins from Plasmodium falciparum that are homologous to reticulocyte binding proteins in Plasmodium vivax.* Infect Immun, 2001. **69**(2): p. 1084-92.

113. Ogun, S.A., et al., *Plasmodium yoelii: effects of red blood cell modification and antibodies on the binding characteristics of the 235-kDa rhoptry protein.* Exp Parasitol, 2000. **95**(3): p. 187-95.

114. Preiser, P.R., et al., *Stage-specific transcription of distinct repertoires of a multigene family during Plasmodium life cycle.* Science, 2002. **295**(5553): p. 342-5.

115. Greenwood, B. and T. Mutabingwa, *Malaria in 2002.* Nature, 2002. **415**(6872): p. 670-672.

116. Tongren, J.E., et al., *Malaria vaccines: if at first you don't succeed.* Trends in Parasitology, 2004. **20**(12): p. 604-610.

117. Nussenzweig, R.S., et al., *Protective immunity produced by the injection of x-irradiated sporozoites of plasmodium berghei.* Nature, 1967. **216**(5111): p. 160-2.

118. Collins, W.E. and P.G. Contacos, *Immunization of monkeys against Plasmodium cynomolgi by x-radiated sporozoites.* Nature-New Biology, 1972. **236**(67): p. 176-&.

119. Clyde, D.F., et al., *Immunization of man against sporzoite-induced falciparum malaria.* American Journal of the Medical Sciences, 1973. **266**(3): p. 169-177.

120. Hoffman, S.L., et al., *Protection of humans against malaria by immunization with radiation-attenuated Plasmodium falciparum sporozoites.* Journal of Infectious Diseases, 2002. **185**(8): p. 1155-1164.

121. Cohen, S., S. Carrington, and I.A. McGregor, *Gamma-globulin and acquired immunity to human malaria.* Nature, 1961. **192**(480): p. 733-&.

122. Sabchareon, A., et al., *Parasitological and clinical human response to immunoglobulin administration in falciparum malaria.* American Journal of Tropical Medicine and Hygiene, 1991. **45**(3): p. 297-308.

123. Kester, K.E., et al., *Efficacy of recombinant circumsporozoite protein vaccine regimens against experimental Plasmodium falciparum malaria.* Journal of Infectious Diseases, 2001. **183**(4): p. 640-647.

124. Bojang, K.A., et al., *Efficacy of RTS,S/ASO2 malaria vaccine against Plasmodium falciparum infection in semi-immune adult men in The Gambia: a randomised trial.* Lancet, 2001. **358**(9297): p. 1927-1934.

125. Alonso, P.L., et al., *Efficacy of the RTS,S/AS02A vaccine against Plasmodium falciparum infection and disease in young African children: randomised controlled trial.* Lancet, 2004. **364**(9443): p. 1411-1420.

126. Bojang, K.A., et al., *Safety and immunogenicty of RTS,S/AS02A candidate malaria vaccine in Gambian children.* Vaccine, 2005. **23**(32): p. 4148-4157.

127. Macete, E., et al., *Safety and immunogenicity of the RTS,S/AS02A candidate malaria vaccine in children aged 1-4 in Mozambique.* Tropical Medicine & International Health, 2007. **12**(1): p. 37-46.

128. Macete, E.V., et al., *Evaluation of two formulations of adjuvanted RTS, S malaria vaccine in children aged 3 to 5 years living in a malaria-endemic region of Mozambique: a Phase I/IIb randomized double-blind bridging trial.* Trials, 2007. **8**.

129. Aponte, J.J., et al., *Safety of the RTS,S/AS02D candidate malaria vaccine in infants living in a highly endemic area of Mozambique: a double blind randomised controlled phase I/IIb trial.* Lancet, 2007. **370**(9598): p. 1543-1551.

130. Alonso, P.L., et al., *Duration of protection with RTS,S/AS02A malaria vaccine in prevention of Plasmodium falciparum disease in Mozambican children: single-blind extended follow-up of a randomised controlled trial.* Lancet, 2005. **366**(9502): p. 2012-2018.

131. Druilhe, P. and J.W. Barnwell, *Pre-erythrocytic stage malaria vaccines: time for a change in path.* Current Opinion in Microbiology, 2007. **10**(4): p. 371-378.

132. Snounou, G., et al., *The Plasmodium sporozoite survives RTS,S vaccination.* Trends in Parasitology, 2005. **21**(10): p. 456-461.

133. Lalvani, A., et al., *Potent induction of focused Th1-type cellular and humoral immune responses by RTS,S/SBAS2, a recombinant Plasmodium falciparum malaria vaccine.* Journal of Infectious Diseases, 1999. **180**(5): p. 1656-1664.

134. Sun, P.F., et al., *Protective immunity induced with malaria vaccine, RTS,S, is linked to Plasmodium falciparum circumsporozoite protein-specific CD4(+) and CD8(+) T cells producing IFN-gamma.* Journal of Immunology, 2003. **171**(12): p. 6961-6967.

135. Gruener, A.C., et al., *Sterile Protection against Malaria Is Independent of Immune Responses to the Circumsporozoite Protein.* PLoS One, 2007. **2**(12).

136. Kumkhaek, C., et al., *Are extensive T cell epitope polymorphisms in the Plasmodium falciparum circumsporozoite antigen, a leading sporozoite vaccine candidate, selected by immune pressure?* Journal of Immunology, 2005. **175**(6): p. 3935-3939.

137. Weedall, G.D., et al., *Differential evidence of natural selection on two leading sporozoite stage malaria vaccine candidate antigens.* International Journal for Parasitology, 2007. **37**(1): p. 77-85.

138. Enosse, S., et al., *RTS,S/AS02A malaria vaccine does not induce parasite CSP T cell epitope selection and reduces multiplicity of infection.* Plos Clinical Trials, 2006. **1**(1).

139. Genton, B. and Z.H. Reed, *Asexual blood-stage malaria vaccine development: facing the challenges.* Current Opinion in Infectious Diseases, 2007. **20**(5): p. 467-475.

140. Genton, B., et al., *A recombinant blood-stage malaria vaccine reduces Plasmodium falciparum density and exerts selective pressure on parasite populations in a phase 1-2b trial in Papua New Guinea.* J Infect Dis, 2002. **185**(6): p. 820-7.

141. Fluck, C., et al., *Strain-specific humoral response to a polymorphic malaria vaccine.* Infection and Immunity, 2004. **72**(11): p. 6300-6305.

142. Wu, Y., et al., *Phase 1 trial of malaria transmission blocking vaccine candidates Pfs25 and Pvs25 formulated with montanide ISA 51.* PLoS One, 2008. **3**(7): p. e2636.

143. Malkin, E.M., et al., *Phase 1 vaccine trial of Pvs25H: a transmission blocking vaccine for Plasmodium vivax malaria.* Vaccine, 2005. **23**(24): p. 3131-3138.

144. Patarroyo, M.E., et al., *Induction of protective immunity against experimental infection with malaria using synthetic peptides.* Nature, 1987. **328**(6131): p. 629-632.

145. Graves, P. and H. Gelband, *Vaccines for preventing malaria (SPf66).* Cochrane Database of Systematic Reviews, 2006(2).

146. Snounou, G. and L. Renia, *The vaccine is dead - long live the vaccine.* Trends in Parasitology, 2007. **23**(4): p. 129-132.

147. Gilbert, S.C. and A.V.S. Hill, *The SPf66 malaria vaccine: Where do we go from here?* Parasitology Today, 1998. **14**(6): p. 251-251.

148. Ferreira, M., *Malaria vaccine trials: The missing qualitative data.* Immunology and Cell Biology, 1996. **74**(4): p. 301-305.

149. Ockenhouse, C.F., et al., *Phase I/IIa safety, immunogenicity, and efficacy trial of NYVAC-Pf7, a pox-vectored, multiantigen, multistage vaccine candidate for Plasmodium falciparum malaria.* Journal of Infectious Diseases, 1998. **177**(6): p. 1664-1673.

150. Trager, W. and J.B. Jensen, *Human malaria parasites in continuous culture.* Science, 1976. **193**(4254): p. 673-675.

151. Chulay, J.D., et al., *Malaria transmitted to humans by mosquitos infected from cultured Plasmodium falciparum.* American Journal of Tropical Medicine and Hygiene, 1986. **35**(1): p. 66-68.

152. Mueller, A.K., et al., *Plasmodium liver stage developmental arrest by depletion of a protein at the parasite-host interface.* Proceedings of the National Academy of Sciences of the United States of America, 2005. **102**(8): p. 3022-3027.

153. van Dijk, M.R., et al., *Genetically attenuated, P36p-deficient malarial sporozoites induce protective immunity and apoptosis of infected liver cells.* Proceedings of the National Academy of Sciences of the United States of America, 2005. **102**(34): p. 12194-12199.

154. Wack, A. and R. Rappuoli, *Vaccinology at the beginning of the 21st century.* Curr Opin Immunol, 2005. **17**(4): p. 411-8.

155. Kenney, R.T. and R. Edelman, *Survey of human-use adjuvants.* Expert Rev Vaccines, 2003. **2**(2): p. 167-88.

156. Coler, R.N., et al., *Adjuvants for malaria vaccines.* Parasite Immunol, 2009. **31**(9): p. 520-8.

157. Reed, S.G., et al., *New horizons in adjuvants for vaccine development.* Trends Immunol, 2009. **30**(1): p. 23-32.

158. Billiau, A. and P. Matthys, *Modes of action of Freund's adjuvants in experimental models of autoimmune diseases.* J Leukoc Biol, 2001. **70**(6): p. 849-60.

159. Cerwenka, A. and L.L. Lanier, *Ligands for natural killer cell receptors: redundancy or specificity.* Immunol Rev, 2001. **181**: p. 158-69.

160. Pozdnyakova, O., et al., *Impaired antibody response to group B streptococcal type III capsular polysaccharide in C3- and complement receptor 2-deficient mice.* J Immunol, 2003. **170**(1): p. 84-90.

161. Prinetti, A., et al., *Immunoseparation of sphingolipid-enriched membrane domains enriched in Src family protein tyrosine kinases and in the neuronal adhesion molecule TAG-1 by anti-GD3 ganglioside monoclonal antibody.* J Neurochem, 2001. **78**(5): p. 1162-7.

162. Sargsyan, E., et al., *Identification of ERp29, an endoplasmic reticulum lumenal protein, as a new member of the thyroglobulin folding complex.* J Biol Chem, 2002. **277**(19): p. 17009-15.

163. Goedert, J.J., C.S. Rabkin, and S.R. Ross, *Prevalence of serologic reactivity against four strains of mouse mammary tumour virus among US women with breast cancer.* Br J Cancer, 2006. **94**(4): p. 548-51.

164. Heegaard, N.H., et al., *Immunoaffinity chromatographic and immunoprecipitation methods combined with mass spectrometry for characterization of circulating transthyretin.* J Sep Sci, 2006. **29**(3): p. 371-7.

165. Rascon, A., et al., *Purification and properties of the cGMP-inhibited cAMP phosphodiesterase from bovine aortic smooth muscle.* Biochim Biophys Acta, 1992. **1134**(2): p. 149-56.

166. Stam, H. and W.C. Hulsmann, *Effects of hormones, amino acids and specific inhibitors on rat heart heparin-releasable lipoprotein lipase and tissue neutral lipase activities during long-term perfusion.* Biochim Biophys Acta, 1984. **794**(1): p. 72-82.

167. Doppler, H., et al., *A phosphorylation state-specific antibody recognizes Hsp27, a novel substrate of protein kinase D.* J Biol Chem, 2005. **280**(15): p. 15013-9.

168. Schneider, R., et al., *Histone H3 lysine 4 methylation patterns in higher eukaryotic genes.* Nat Cell Biol, 2004. **6**(1): p. 73-7.

169. Marcora, E., K. Gowan, and J.E. Lee, *Stimulation of NeuroD activity by huntingtin and huntingtin-associated proteins HAP1 and MLK2.* Proc Natl Acad Sci U S A, 2003. **100**(16): p. 9578-83.

170. Monferran, S., et al., *The membrane form of the DNA repair protein Ku interacts at the cell surface with metalloproteinase 9.* EMBO J, 2004. **23**(19): p. 3758-68.

171. Gridley, S., et al., *Novel insulin-elicited phosphoproteins in adipocytes.* Cell Signal, 2005. **17**(1): p. 59-66.

172. Schilling, B., et al., *Proteomic analysis of succinate dehydrogenase and ubiquinol-cytochrome c reductase (Complex II and III) isolated by immunoprecipitation from bovine and mouse heart mitochondria.* Biochim Biophys Acta, 2006. **1762**(2): p. 213-22.

173. Barnouin, K., *Two-dimensional gel electrophoresis for analysis of protein complexes.* Methods Mol Biol, 2004. **261**: p. 479-98.

174. Anzai, N., et al., *The multivalent PDZ domain-containing protein PDZK1 regulates transport activity of renal urate-anion exchanger URAT1 via its C terminus.* J Biol Chem, 2004. **279**(44): p. 45942-50.

175. Faber, E.S., et al., *Synaptic activation of transient receptor potential channels by metabotropic glutamate receptors in the lateral amygdala.* Neuroscience, 2006. **137**(3): p. 781-94.

176. Qoronfleh, M.W., et al., *Use of Immunomatrix Methods to Improve Protein-Protein Interaction Detection.* J Biomed Biotechnol, 2003. **2003**(5): p. 291-298.

177. Khundmiri, S.J., M.J. Rane, and E.D. Lederer, *Parathyroid hormone regulation of type II sodium-phosphate cotransporters is dependent on an A kinase anchoring protein.* J Biol Chem, 2003. **278**(12): p. 10134-41.

178. Sisson, T.H. and C.W. Castor, *An improved method for immobilizing IgG antibodies on protein A-agarose.* J Immunol Methods, 1990. **127**(2): p. 215-20.

179. Domen, P.L., et al., *Site-directed immobilization of proteins.* J Chromatogr, 1990. **510**: p. 293-302.

180. Seko, Y., et al., *Selective cytoplasmic translocation of HuR and site-specific binding to the interleukin-2 mRNA are not sufficient for CD28-mediated stabilization of the mRNA.* J Biol Chem, 2004. **279**(32): p. 33359-67.

181. Kaboord, B. and M. Perr, *Isolation of proteins and protein complexes by immunoprecipitation.* Methods Mol Biol, 2008. **424**: p. 349-64.

182. Chen, Y.H. and Q. Lu, *Association of nonreceptor tyrosine kinase c-yes with tight junction protein occludin by coimmunoprecipitation assay.* Methods Mol Biol, 2003. **218**: p. 127-32.

183. Loven, M.A., et al., *A novel estrogen receptor alpha-associated protein alters receptor-deoxyribonucleic acid interactions and represses receptor-mediated transcription.* Mol Endocrinol, 2004. **18**(11): p. 2649-59.

184. Mendez, D., et al., *Stress response and cytoskeletal proteins involved in erythrocyte membrane remodeling upon Plasmodium falciparum invasion are differentially carbonylated in G6PD A- deficiency.* Free Radic Biol Med, 2011. **50**(10): p. 1305-13.

185. Mendez, D., et al., *Differential carbonylation of cytoskeletal proteins in blood group O erythrocytes: Potential role in protection against severe malaria.* Infect Genet Evol, 2012. **12**(8): p. 1780-1787.

186. Davies, M.J., et al., *Stable markers of oxidant damage to proteins and their application in the study of human disease.* Free Radic Biol Med, 1999. **27**(11-12): p. 1151-63.

187. Beckman, K.B. and B.N. Ames, *The free radical theory of aging matures.* Physiol Rev, 1998. **78**(2): p. 547-81.

188. Berlett, B.S. and E.R. Stadtman, *Protein oxidation in aging, disease, and oxidative stress.* J Biol Chem, 1997. **272**(33): p. 20313-6.

189. Ghezzi, P. and V. Bonetto, *Redox proteomics: identification of oxidatively modified proteins.* Proteomics, 2003. **3**(7): p. 1145-53.

190. Stadtman, E.R. and R.L. Levine, *Protein oxidation.* Ann N Y Acad Sci, 2000. **899**: p. 191-208.

191. Butterfield, D.A. and J. Kanski, *Brain protein oxidation in age-related neurodegenerative disorders that are associated with aggregated proteins.* Mech Ageing Dev, 2001. **122**(9): p. 945-62.

192. Dalle-Donne, I., et al., *Protein carbonylation, cellular dysfunction, and disease progression.* J Cell Mol Med, 2006. **10**(2): p. 389-406.

193. Levine, R.L., et al., *Determination of carbonyl content in oxidatively modified proteins.* Methods Enzymol, 1990. **186**: p. 464-78.

194. Levine, R.L., et al., *Carbonyl assays for determination of oxidatively modified proteins.* Methods Enzymol, 1994. **233**: p. 346-57.

195. Talent, J.M., Y. Kong, and R.W. Gracy, *A double stain for total and oxidized proteins from two-dimensional fingerprints.* Anal Biochem, 1998. **263**(1): p. 31-8.

196. Robinson, C.E., et al., *Determination of protein carbonyl groups by immunoblotting.* Anal Biochem, 1999. **266**(1): p. 48-57.

197. Cao, G. and R.G. Cutler, *Protein oxidation and aging. II. Difficulties in measuring alkaline protease activity in tissues using the fluorescamine procedure.* Arch Biochem Biophys, 1995. **320**(1): p. 195-201.

198. Cao, G. and R.G. Cutler, *Protein oxidation and aging. I. Difficulties in measuring reactive protein carbonyls in tissues using 2,4-dinitrophenylhydrazine.* Arch Biochem Biophys, 1995. **320**(1): p. 106-14.

199. Utrera, M., et al., *Fluorescent HPLC for the detection of specific protein oxidation carbonyls - alpha-aminoadipic and gamma-glutamic semialdehydes - in meat systems.* Meat Sci, 2011. **89**(4): p. 500-6.

200. Agarwal, S. and R.S. Sohal, *Differential oxidative damage to mitochondrial proteins during aging.* Mech Ageing Dev, 1995. **85**(1): p. 55-63.

201. Keller, R.J., et al., *Immunochemical detection of oxidized proteins.* Chem Res Toxicol, 1993. **6**(4): p. 430-3.

202. Shacter, E., et al., *Differential susceptibility of plasma proteins to oxidative modification: examination by western blot immunoassay.* Free Radic Biol Med, 1994. **17**(5): p. 429-37.

203. Conrad, C.C., et al., *Post-Electrophoretic Identification of Oxidized Proteins.* Biol Proced Online, 2000. **2**: p. 39-45.

204. Linares, M., et al., *Proteomic approaches to identifying carbonylated proteins in brain tissue.* J Proteome Res, 2011. **10**(4): p. 1719-27.

205. Radfar, A., A. Diez, and J.M. Bautista, *Chloroquine mediates specific proteome oxidative damage across the erythrocytic cycle of resistant Plasmodium falciparum.* Free Radic Biol Med, 2008. **44**(12): p. 2034-42.

206. Comporti, M., *Lipid peroxidation and biogenic aldehydes: from the identification of 4-hydroxynonenal to further achievements in biopathology.* Free Radic Res, 1998. **28**(6): p. 623-35.

207. Esterbauer, H., R.J. Schaur, and H. Zollner, *Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes.* Free Radic Biol Med, 1991. **11**(1): p. 81-128.

208. Chavez, J., et al., *Site-specific protein adducts of 4-hydroxy-2(E)-nonenal in human THP-1 monocytic cells: protein carbonylation is diminished by ascorbic acid.* Chem Res Toxicol, 2010. **23**(1): p. 37-47.

209. Guo, J., et al., *Protein targets for carbonylation by 4-hydroxy-2-nonenal in rat liver mitochondria.* J Proteomics, 2011. **74**(11): p. 2370-9.

210. Madian, A.G. and F.E. Regnier, *Proteomic identification of carbonylated proteins and their oxidation sites.* J Proteome Res, 2010. **9**(8): p. 3766-80.

211. Roe, M.R. and T.J. Griffin, *Gel-free mass spectrometry-based high throughput proteomics: tools for studying biological response of proteins and proteomes.* Proteomics, 2006. **6**(17): p. 4678-87.

212. Tanito, M., et al., *Identification of 4-hydroxynonenal-modified retinal proteins induced by photooxidative stress prior to retinal degeneration.* Free Radic Biol Med, 2006. **41**(12): p. 1847-59.

213. Laurent, A., et al., *Metabolism of 4-hydroxynonenal, a cytotoxic product of lipid peroxidation, in rat precision-cut liver slices.* Toxicol Lett, 2000. **114**(1-3): p. 203-14.

214. Srivastava, S., et al., *Metabolism of lipid peroxidation product, 4-hydroxynonenal (HNE) in rat erythrocytes: role of aldose reductase.* Free Radic Biol Med, 2000. **29**(7): p. 642-51.

215. Uchida, K. and E.R. Stadtman, *Modification of histidine residues in proteins by reaction with 4-hydroxynonenal.* Proc Natl Acad Sci U S A, 1992. **89**(10): p. 4544-8.

216. Nadkarni, D.V. and L.M. Sayre, *Structural definition of early lysine and histidine adduction chemistry of 4-hydroxynonenal.* Chem Res Toxicol, 1995. **8**(2): p. 284-91.

217. Szweda, L.I., et al., *Inactivation of glucose-6-phosphate dehydrogenase by 4-hydroxy-2-nonenal. Selective modification of an active-site lysine.* J Biol Chem, 1993. **268**(5): p. 3342-7.

218. Sayre, L.M., et al., *Pyrrole formation from 4-hydroxynonenal and primary amines.* Chem Res Toxicol, 1993. **6**(1): p. 19-22.

219. Pan, J. and F.L. Chung, *Formation of cyclic deoxyguanosine adducts from omega-3 and omega-6 polyunsaturated fatty acids under oxidative conditions.* Chem Res Toxicol, 2002. **15**(3): p. 367-72.

220. Zhou, S. and E.A. Decker, *Ability of carnosine and other skeletal muscle components to quench unsaturated aldehydic lipid oxidation products.* J Agric Food Chem, 1999. **47**(1): p. 51-5.

221. Guichardant, M., et al., *Covalent modifications of aminophospholipids by 4-hydroxynonenal.* Free Radic Biol Med, 1998. **25**(9): p. 1049-56.

222. Carini, M., G. Aldini, and R.M. Facino, *Mass spectrometry for detection of 4-hydroxy-trans-2-nonenal (HNE) adducts with peptides and proteins.* Mass Spectrom Rev, 2004. **23**(4): p. 281-305.

223. Cohn, J.A., et al., *Chemical characterization of a protein-4-hydroxy-2-nonenal cross-link: immunochemical detection in mitochondria exposed to oxidative stress.* Arch Biochem Biophys, 1996. **328**(1): p. 158-64.

224. Roque, A.C., G. Gupta, and C.R. Lowe, *Design, synthesis, and screening of biomimetic ligands for affinity chromatography.* Methods Mol Biol, 2005. **310**: p. 43-62.

225. Follman, D.K. and R.L. Fahrner, *Factorial screening of antibody purification processes using three chromatography steps without protein A.* J Chromatogr A, 2004. **1024**(1-2): p. 79-85.

226. Firer, M.A., *Efficient elution of functional proteins in affinity chromatography.* J Biochem Biophys Methods, 2001. **49**(1-3): p. 433-42.

227. Cuatrecasas, P. and M. Wilchek, *Single-step purification of avidine from egg white by affinity chromatography on biocytin-Sepharose columns.* Biochem Biophys Res Commun, 1968. **33**(2): p. 235-9.

228. Cuatrecasas, P., M. Wilchek, and C.B. Anfinsen, *Selective enzyme purification by affinity chromatography.* Proc Natl Acad Sci U S A, 1968. **61**(2): p. 636-43.

229. Ghose, S., B. Hubbard, and S.M. Cramer, *Evaluation and comparison of alternatives to Protein A chromatography Mimetic and hydrophobic charge induction chromatographic stationary phases.* J Chromatogr A, 2006. **1122**(1-2): p. 144-52.

230. Roque, A.C., C.S. Silva, and M.A. Taipa, *Affinity-based methodologies and ligands for antibody purification: advances and perspectives.* J Chromatogr A, 2007. **1160**(1-2): p. 44-55.

231. Fassina, G., et al., *Novel ligands for the affinity-chromatographic purification of antibodies.* J Biochem Biophys Methods, 2001. **49**(1-3): p. 481-90.

232. Hahn, R., et al., *Comparison of protein A affinity sorbents III. Life time study.* J Chromatogr A, 2006. **1102**(1-2): p. 224-31.

233. Hedhammar, M., et al., *Single-step recovery and solid-phase refolding of inclusion body proteins using a polycationic purification tag.* Biotechnol J, 2006. **1**(2): p. 187-96.

234. Low, D., R. O'Leary, and N.S. Pujar, *Future of antibody purification.* J Chromatogr B Analyt Technol Biomed Life Sci, 2007. **848**(1): p. 48-63.

235. Nisnevitch, M. and M.A. Firer, *The solid phase in affinity chromatography: strategies for antibody attachment.* J Biochem Biophys Methods, 2001. **49**(1-3): p. 467-80.

236. Bergmann-Leitner, E.S., et al., *Evaluation of immunoglobulin purification methods and their impact on quality and yield of antigen-specific antibodies.* Malar J, 2008. **7**: p. 129.

237. Bjorck, L. and G. Kronvall, *Purification and some properties of streptococcal protein G, a novel IgG-binding reagent.* J Immunol, 1984. **133**(2): p. 969-74.

238. Dancette, O.P., et al., *Purification of immunoglobulins G by protein A/G affinity membrane chromatography.* J Chromatogr B Biomed Sci Appl, 1999. **723**(1-2): p. 61-8.

239. Targett, G.A., *Malaria vaccines 1985-2005: a full circle?* Trends Parasitol, 2005. **21**(11): p. 499-503.

240. Aguiar, J.C., et al., *High-throughput generation of P. falciparum functional molecules by recombinational cloning.* Genome Res, 2004. **14**(10B): p. 2076-82.

241. Gardner, M.J., et al., *Genome sequence of the human malaria parasite Plasmodium falciparum.* Nature, 2002. **419**(6906): p. 498-511.

242. Good, M.F., *Vaccine-induced immunity to malaria parasites and the need for novel strategies.* Trends Parasitol, 2005. **21**(1): p. 29-34.

243. Rappuoli, R. and A. Aderem, *A 2020 vision for vaccines against HIV, tuberculosis and malaria.* Nature, 2011. **473**(7348): p. 463-9.

244. Dutta, S., et al., *Structural basis of antigenic escape of a malaria vaccine candidate.* Proc Natl Acad Sci U S A, 2007. **104**(30): p. 12488-93.

245. Kusi, K.A., et al., *Humoral immune response to mixed PfAMA1 alleles; multivalent PfAMA1 vaccines induce broad specificity.* PLoS One, 2009. **4**(12): p. e8110.

246. Remarque, E.J., et al., *A diversity-covering approach to immunization with Plasmodium falciparum apical membrane antigen 1 induces broader allelic recognition and growth inhibition responses in rabbits.* Infect Immun, 2008. **76**(6): p. 2660-70.

247. Scarselli, M., et al., *Rational design of a meningococcal antigen inducing broad protective immunity.* Sci Transl Med, 2011. **3**(91): p. 91ra62.

248. Martinelli, A., et al., *A genetic approach to the de novo identification of targets of strain-specific immunity in malaria parasites.* Proc Natl Acad Sci U S A, 2005. **102**(3): p. 814-9.

249. Gruner, A.C., et al., *Pre-erythrocytic antigens of Plasmodium falciparum: from rags to riches?* Trends Parasitol, 2003. **19**(2): p. 74-8.

250. Oeuvray, C., et al., *Merozoite surface protein-3: a malaria protein inducing antibodies that promote Plasmodium falciparum killing by cooperation with blood monocytes.* Blood, 1994. **84**(5): p. 1594-602.

251. Bull, P.C., et al., *Plasmodium falciparum-infected erythrocytes: agglutination by diverse Kenyan plasma is associated with severe disease and young host age.* J Infect Dis, 2000. **182**(1): p. 252-9.

252. Stanisic, D.I., et al., *Analysis of immunological nonresponsiveness to the 19-kilodalton fragment of merozoite surface Protein 1 of Plasmodium yoelii: rescue by chemical conjugation to diphtheria toxoid (DT) and enhancement of immunogenicity by prior DT vaccination.* Infect Immun, 2003. **71**(10): p. 5700-13.

253. Pombo, D.J., et al., *Immunity to malaria after administration of ultra-low doses of red cells infected with Plasmodium falciparum.* Lancet, 2002. **360**(9333): p. 610-7.

254. Makobongo, M.O., et al., *The purine salvage enzyme hypoxanthine guanine xanthine phosphoribosyl transferase is a major target antigen for cell-mediated immunity to malaria.* Proc Natl Acad Sci U S A, 2003. **100**(5): p. 2628-33.

255. Yoeli, M., *Patterns of immunity and resistance in rodent malaria infections.* Bull Soc Pathol Exot Filiales, 1966. **59**(4): p. 593-605.

256. Cox, F.E., *Acquired immunity to Plasmodium vinckei in mice.* Parasitology, 1966. **56**(4): p. 719-32.

257. Cox, H.W. and R. Milar, *Cross-protection immunization by Plasmodium and Babesia infections of rats and mice.* Am J Trop Med Hyg, 1968. **17**(2): p. 173-9.

258. McColm, A.A. and L. Dalton, *Heterologous immunity in rodent malaria: comparison of the degree of cross-immunity generated by vaccination with that produced by exposure to live infection.* Ann Trop Med Parasitol, 1983. **77**(4): p. 355-77.

259. Grun, J.L. and W.P. Weidanz, *Antibody-independent immunity to reinfection malaria in B-cell-deficient mice.* Infect Immun, 1983. **41**(3): p. 1197-204.

260. Jarra, W. and K.N. Brown, *Protective immunity to malaria: studies with cloned lines of Plasmodium chabaudi and P. berghei in CBA/Ca mice. I. The effectiveness and inter- and intra-species specificity of immunity induced by infection.* Parasite Immunol, 1985. **7**(6): p. 595-606.

261. Legorreta-Herrera, M., et al., *Early treatment during a primary malaria infection modifies the development of cross immunity.* Parasite Immunol, 2004. **26**(1): p. 7-17.

262. Elliott, S.R., R.D. Kuns, and M.F. Good, *Heterologous immunity in the absence of variant-specific antibodies after exposure to subpatent infection with blood-stage malaria.* Infect Immun, 2005. **73**(4): p. 2478-85.

263. Belnoue, E., et al., *Vaccination with live Plasmodium yoelii blood stage parasites under chloroquine cover induces cross-stage immunity against malaria liver stage.* J Immunol, 2008. **181**(12): p. 8552-8.

264. Renia, L., *Protective immunity against malaria liver stage after vaccination with live parasites.* Parasite, 2008. **15**(3): p. 379-83.

265. D'Antonio, L.E., et al., *Malaria resistance: artificial induction with a partially purified plasmodial fraction.* Science, 1970. **168**(3935): p. 1117-8.

266. Hommel, M., et al., *Protection against Plasmodium chabaudi malaria. I.--Vaccination of mice with merozoites and Freund's adjuvants.* Ann Immunol (Paris), 1982. **133C**(1): p. 57-67.

267. Su, Z., et al., *Vaccination with novel immunostimulatory adjuvants against blood-stage malaria in mice.* Infect Immun, 2003. **71**(9): p. 5178-87.

268. Wellde, B.T. and E.H. Sadun, *Resistance produced in rats and mice by exposure to irradiated Plasmodium berghei.* Exp Parasitol, 1967. **21**(3): p. 310-24.

269. Wellde, B.T., R.A. Ward, and R. Ueoka, *Aspects of immunity in mice inoculated with irradiated Plasmodium berghei.* Mil Med, 1969. **134**(10): p. 1153-64.

270. Waki, S., et al., *Plasmodium berghei: isolation and maintenance of an irradiation attenuated strain in the nude mouse.* Exp Parasitol, 1982. **53**(3): p. 335-40.

271. Waki, S., I. Yonome, and M. Suzuki, *Plasmodium yoelii: induction of attenuated mutants by irradiation.* Exp Parasitol, 1986. **62**(3): p. 316-21.

272. Ting, L.M., et al., *Attenuated Plasmodium yoelii lacking purine nucleoside phosphorylase confer protective immunity.* Nat Med, 2008. **14**(9): p. 954-8.

273. Roestenberg, M., et al., *Protection against a malaria challenge by sporozoite inoculation.* N Engl J Med, 2009. **361**(5): p. 468-77.

274. Struik, S.S. and E.M. Riley, *Does malaria suffer from lack of memory?* Immunol Rev, 2004. **201**: p. 268-90.